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Review

Development of the visual system of the chick II. Mechanisms of axonal guidance

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Abstract

The quest to understand axonal guidance mechanisms requires exact and multidisciplinary analyses of axon navigation. This review is the second part of an attempt to synthesise experimental data with theoretical models of the development of the topographic connection of the chick retina with the tectum. The first part included classic ideas from developmental biology and recent achievements on the molecular level in understanding cytodifferentiation and histogenesis [J. Mey, S. Thanos, Development of the visual system of the chick. (I) Cell differentiation and histogenesis, Brain Res. Rev. 32 (2000) 343–379]. The present part deals with the question of how millions of fibres exit from the eye, traverse over several millimetres and spread over the optic tectum to assemble a topographic map, whose precision accounts for the sensory performance of the visual system. The following topics gained special attention in this review. (i) A remarkable conceptual continuity between classic embryology and recent molecular biology has revealed that positional cellular specification precedes and determines the formation of the retinotectal map. (ii) Graded expression of asymmetric genes, transcriptional factors and receptors for signal transduction during early development seem to play a crucial role in determining the spatial identity of neurons within surface areas of retina and optic tectum. (iii) The chemoaffinity hypothesis constitutes the conceptual framework for development of the retinotopic organisation of the primary visual pathway. Studies of repulsive factors in vitro developed the original hypothesis from a theoretical postulate of chemoattraction to an empirically supported concept based on chemorepulsion. (iv) The independent but synchronous development of retina and optic tectum in topo-chronologically corresponding patterns ensures that ingrowing retinal axons encounter receptive target tissue at appropriate locations, and at the time when connections are due to be formed. (v) The growth cones of the retino-fugal axons seem to be guided both by local cues on glial endfeet and within the extracellular matrix. On the molecular level, the ephrins and their receptors have emerged as the most likely candidates for the material substrate of a topographic projection along the anterior-posterior axis of the optic tectum. Yet, since a number of alternative molecules have been proposed for the same function, it remains the challenge for the near future to define the proportional contribution of each one of the individual mechanisms proposed by matching theoretical predictions with the experimental evidence.

Keywords: Retina; Tectum; Chick; Development; Visual system; Retinotopy; Axonal guidance; Histogenesis

Abbreviations: AChE, acetylcholine esterase; AChr-β2, acetylcholin receptor-beta 2; ALDH, aldehyde dehydrogenase; BDNF, brain-derived neurotrophic factor; BM, basement membrane; CAMP, cyclic adenosine monophosphate; CBF-1, CBF-2, chicken brain factor-1 and 2; ChAT, choline acetyltransferase; CNS, central nervous system; CNTF, ciliary neurotrophic factor; DHD-2, dialdehyde-dededrogenase-2; DiI, 1,1′-dioctadecyl-3,3,3,′3′-tetramethyl-indocarbocyanine perchlorate; E (followed by number), embryonic day; ECM, extracellular matrix; EGF, epidermal growth factor; ELF-1, eph ligand; *en*, engrailed; *Eya*, eye absent; FGF-8, fibroblast growth factor 8; GABA, gamma-amino butyric acid; GAD, glutamic acid decarboxylase; GAP, growth associated protein; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; GTX-I, grayanotoxin-I; HH-1, Hamburger-Hamilton stage 1 (chick embryo staging table); *hox*, homeobox; HRP, horseradish peroxidase (neuroanatomical tracer); INL, inner nuclear layer; IPL, inner plexiform layer; N-CAM, neural cell adhesion molecule; Ng-CAM, neuroglial cell adhesion molecule; NGF, nerve growth factor; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; NT, neurotrophin; OFL, optic fibre layer; *Pax-2, Pax-6*, paired box genes; PG, proteoglycan; PI-PLP, phosphatidyl inositol-phospholipase; PM-1, precursor marker 1; RA, retinoic acid; RAGS, repulsive axon guidance signal (Ephrin-A5); rER, rough endoplasmic reticulum; RGC, retinal ganglion cell; RGM, repulsive guidance molecule; RITC, rhodamin-B-isothiocyanate (fluorescent tracer); RPTP, receptor tyrosine phosphatase; SAC, stratum album centrale (of the optic tectum); *Sey*, small eye; SFP, stratum fibrosum periventriculare (of the tectum); SGC, stratum griseum centrale (of the tectum); SGFS, stratum griseum et fibrosum superficiale (of the tectum); SGP, stratum griseum periventriculare (of the tectum); SO, stratum opticum (of the tectum); *SOHO-1*, sonic hedgehoc gene 1; SP, substance P (neurotransmitter); Tbx-5, T-box-transcription factor gene 5; TOP_{AP}, toponymic anterior-posterior; TOP_{DV}, toponymic dorsal-ventral; TrkA, B, C, tyrosine kinase A, B, C (neurotrophin receptors); TTX, tetrodotoxin; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling; *Wnt*, wingless

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Contents

1. Introduction

In the relatively short period of a few decades, the chick visual system has proceeded from a descriptive embryological model, to being recognised as one of the most suitable tools to study molecular mechanisms of neural development and connectivity (Table 1). Like all birds examined so far, chickens possess highly developed visual performance, although their primary visual pathway is comprised of relatively few compartments like the retina, optic tectum and a few thalamic nuclei with secondary connections to other centres. The outstanding cellular complexity of this system is matched by a highly differentiated connectivity of specialised subsets of cellular elements. The ganglion cell layer of the chick retina, for instance, contains ~2.4 million ganglion cells of at least eight different morphological and perhaps physiological types, more than in any other class of vertebrates studied so far. In comparison, the human retina consists of ~1.5 million ganglion cells, and the widely used rat retina consists of only 100 thousand ganglion cells divided into three classes, while the mouse optic nerve consists of even fewer axons ranging from 40 000 to 80 000.

One of the marked features of the chick eye, equally advantageous for embryologists and molecular biologists, is the experimental accessibility from the earliest stages of, and throughout, development. The first compartmentalisation of the visual system is visible after ~30 h of incubation, when the prosencephalic neural tube protrudes laterally to form the optic vesicles, and development is mainly completed at the time of hatching [146,258]. The mature retinotectal connection projects the retinal surfaces onto the surfaces of the contralateral tecta in a precise topological order, thereby preserving neighbourhood relationships between ganglion cells in the distribution of their central contacts. The topography is basically similar to the visual projections in other vertebrates, and more precise than other sensory projections which are also somatotopi-

Table 1
Some landmarks in the study of the retinotectal system in the chick

Time	Advance in understanding of retinotectal development
19th Century	Anatomical description of the avian retinotectal projection
1930s, 1940s	Theoretical models for topographic connections: resonance principle, chemoaffinity hypothesis
Since the 1950s	Tranplantations and lesions in vivo
1970s, 1980s	Neuroanatomical tracing studies
1980s	Theoretical elaborations of the chemoaffinity hypothesis and competing theories
1980s	Search for guidance molecules using bioassays like the stripe assay and the production of antibodies against graded molecules
1995	Identification of Eph receptors and ephrins as guidance molecules in the retinotectal system
Late 1990s, current research	Unravelling spatial polarity and pathfinding with the tools of molecular biology
Late 1990s, current research	Molecular research on intracellular signals in growth cone guidance

cally or tonotopically organised. The chick visual system still serves as an outstanding model to tackle fundamental questions in brain development. In a preceding review we focused on the histogenesis of the major compartments of the visual system and on the underlying molecular mechanisms. In addition to these processes which resemble events in the growth of other organs, one major problem is specific to the nervous system, the establishment of specific connections between neurons.

Ganglion cells axons grow out and are directed towards the optic fissure to form the optic nerve, thereby preserving the positional information of their intraretinal origin. Several research programs and experimental variations are devoted to unravelling the mechanisms of intraretinal fibre growth, guidance, and interaction with cells along this target-independent pathway.

Retinal fibres are first anatomically rearranged into a tube-like order within the optic nerve. When passing through the optic chiasm they interact with a scaffold of local cells in the midline and with axons arriving from the contralateral eye.

To reveal molecular mechanisms of these interactions, overexpression studies can be performed by injections of viral constructs into the neural tube or into the primary optic vesicle at primitive stages, and the disturbing effects of an increasing number of molecules, genes and transcriptional factors can be assessed by neuroanatomical tracing at later stages of development.

Organ cultures are easily prepared both from retina and tectum, and many of the developmental events of the retinotectal topography can be recapitulated in vitro. Consequently, a substantial portion of our current knowledge on axonal growth and guidance is based on these studies.

Finally, although the problem of the retinotectal specificity has long since attracted scientific attention, the molecular processes that confer the necessary information for establishing specific contacts between retinal axons and their central target cells are still a matter of controversy.

The goal of this article is to review the accumulated knowledge on mechanisms of axonal pathfinding in the chick visual system and to provide an introduction for scientists new to the field. This knowledge has been drastically enriched by the advent of new molecular biology techniques, biochemical methods and cell biology approaches needing light into some of the molecular events associated with retinotectal topography. Like the earlier article, which discussed histogenesis and proliferation [221], this paper is also a synthesis of classical and molecular tools in developmental neurobiology. In Section 2 we describe the outcome of the developmental history of the system, namely the topographical properties and the functional performance of the chick visual system. Section 3 reviews the developmental events leading to the mature retinotectal projection. We then, in Section 4, focus on experimental approaches to unravelling molecular mechanisms of these processes and, in Section 5, theoretical models are compared with the empirical evidence.

2. Topographic representation of the visual field

The primary retinotectal projection of chicken like that of all other vertebrates maintains a specific, topographic order at structural levels. This topography preserves the positional relationships between the retinal ganglion cells (RGC) in their central projections. Typically, the retinal images are projected onto the contralateral tectum (Fig. 1) such that the dorsal visual field is represented in the dorsal tectum and the ventral visual field is represented in the ventral tectum. The nasal visual field is projected onto the anterior (rostral) tectum and the temporal visual field is projected onto the posterior (caudal) tectum. Due to the inversion of the image on the retina, the retinal neuronal quadrants are represented in a mirror-imaged fashion onto the corresponding tectal quadrants. This pattern of connectivity displays first a point-to-point projection, and second a central-peripheral projection as indicated in Fig. 1. The projection of the ‘blind-spot’ on the tectum is virtual, because it is not perceptible due to intraretinal mechanisms of integration [271]. This topographical pattern was discovered by electrophysiological methods in pigeon [147], and with anatomical methods in chick [57,66,67]. Since that time the chick retinotectal system has represented a central model in developmental biology, concerning the molecules that guide axons from specific retinal areas to corresponding tectal places.

Due to the lateral position of their eyes, mature chickens

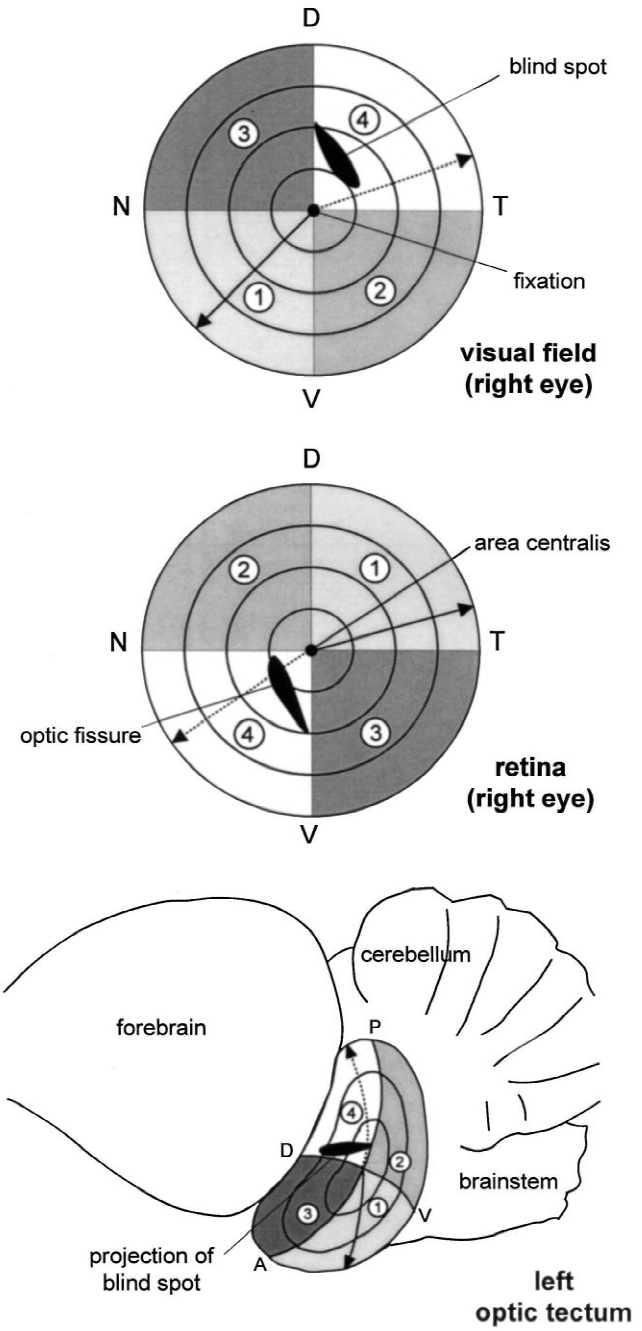


Fig. 1. Schematic drawings showing the representation of the visual field (top) onto the retina (middle) and of the retina onto the tectum. The projection is mirror-imaged along both coordinates, while central to peripheral eccentricity is also preserved as indicated by the arrow. D, dorsal; N, nasal; T, temporal; V, ventral.

have a completely crossed retinofugal pathway. The system is considered functioning and mature from the day of hatching, that is after an incubation of 21 days. In analogy to human vision, the quality of vision or visual performance is described by the parameters visual acuity, extension of the visual field, colour vision, refractive state, binocular vision, stereopsis, and detection of movement. However, although these abilities are closely associated with each other, only visual acuity seems directly related to topographic precision.

As mentioned above, the visual field is almost panoramic in the chicken due to the lateral location of the eyes. However, the frontal visual field shows an overlap of ~25° whereas the optical axes form an angle of 65° with the body axis [356]. It is expected that the visual field has similar characteristics as that of humans. Although not accurately measured yet, the chicken visual field extends like pigeons over ~169° (measured in pigeons) [206,207]. Receptive field properties have been best characterised in anaesthetised, paralysed chicken by recording of single units [370]. More than 60% of the cells were activated by visual targets in the contralateral eye, whereas binocular cells were extremely rare, and cells driven exclusively from the ipsilateral eye were not found. This is in accordance with the fact that the functioning retinofugal projection is completely crossed to the contralateral side [221]. Some regional specializations across the retina account for particular functions of these areas: the ventral half of the retina contains tyrosine hydroxylase type 2 amacrine cells which have been proposed to be associated with the specialisation of this retinal half to detect predators within the upper half of the visual field [71]. This distribution may explain Polyak's [264] description of the chicken behaviour while intensely fixing predators. According to Polyak's description [264], diurnal species of birds have an excellent and well-developed vision both in acuity and stereopsis, although stereopsis is often debated in the chicken. Domestic chickens utilise only one eye to intensely fixate an object, for instance a predator, that they follow with the fixating eye keeping their heads tilted at a slant. In contrast, when picking grain form the floor they use both eyes binocularly and apparently stereoscopically. Visual performance of chickens is reduced in darkness when they become night-blind when objects still remain tolerably distinguishable to human vision.

The visual acuity describes the ability of the retina to resolve high spatial frequencies at the photoreceptor size level and to discriminate two neighbouring signals (resolution). The human visual acuity is ~30–40 cycles/degree and can be determined with subjective and objective methods. Measurement of visual acuity is, however, tedious in the chick. Over and Moore [256] modified the so-called 'preferential looking' methods and determined the highest spatial acuity at which chickens aged 1–25 days jumped consistently to a physically supported grating when they had the choice of an unsupported homogeneous field. The chickens exhibit an asymptotic sensitivity to spatial frequency of ~1.5 cycles/degree and develop this acuity rapidly, that is within the first 2 days of posthatching life. This rapid establishment stands in contrast to a gradually developing visual acuity found for humans, kittens, monkeys and other altricial species. It demonstrates that most of the developmental events occur during embryonic development of the precocial bird species.

Compared to pigeons which have a visual acuity of ~7.1±1.5 cycles/degree [155,265] chickens seem to have a more accurate vision. Although there are no direct studies to show whether the visual acuity changes across the visual field of chickens, studies in pigeons show that the frontal visual field exhibits much higher acuity than the lateral one [144]. There is, however, a marked difference between pigeons and chickens with respect to changes of visual acuity that improve in older pigeons [265]. Whether or not environmental factors determine visual acuity has not been studied in chickens but proposed for falcons [83]. It is expected that the high resolution of the chick retina is based on a precise topographic presentation to the brain, comparable to that of mammals.

In accordance with rapid development of the visual acuity, the refractive state of the chicken eye is quite plastic and can changed by as much as 7 diopters by pushing the retina forward or pulling it back. These movements are possible by changing the thickness of the choroid, the subretinal vascular tissue and the pigment epithelium [357,367]. The elongation of the eye underlies a diurnal rhythm that may be changed experimentally in visually deprived animals [247,295]. The cellular and molecular control of the ocular length plasticity involves synthesis of glycosaminoglycans [246,295] which appeared to determine the susceptibility to deprivation myopia. In a number of experimental set-ups, Schaeffel [294] could show that the control of eye growth is independent of the brain and only obeys local molecular changes. This peculiar ability of the chicken eye is not limited to the retina or the axial length. Also the corneal curvature, and hence the accommodation, can change by up to 9 diopters due to changing intraocular pressure [166]. The association of the refractive state to the mechanisms of retinotopic map formation is, however, not experimentally proven, and theoretically less likely.

3. Ontogenetic course of axonal growth towards the tectum

3.1. Formation of eye cup, polarisation of ganglion cells and positional specification

As in all vertebrates, the eye primordia arise as lateral protrusions from the prosencephalon, in the chick at ~30 h of incubation. As the eye vesicles approach the ectoderm, they induce invagination of the lens placode and initiate the secondary invagination of the eye primordium to form the cup-like eye. These processes are controlled by the *Pax* family of genes, and in particular of *Pax-6*, a homologue to *eyeless* gene in *Drosophila* [215]. Absence of *Pax-6* results in eye malformations. In addition to *Pax-6*, an increasing number of other genes encoding for transcription factors have been discovered which all appear to regulate proliferation and formation of the retinal cell types. Some of these factors, but not all, have also been identified in the chick retina [13].

A distinguishing feature of all species studied so far is the regional specialisation of the retina, which is the structural correlate of particular functions, not all of them understood. For example, a centrally located structure in the chick retina is the so-called area centralis [230], a homologue to the primate fovea centralis [264], and responsible for object fixation and visual acuity. The area centralis is characterised by high density of RGC and different photoreceptor ratios: double cones outnumber rods and single cones 14:10:7, respectively, while in all other fields of the retina the proportion is 2:2:1 [225]. For comparison, in the human fovea centralis cones outnumber rods by roughly 20:1. Topologically, the area centralis of the chicken is localised temporal to the dorsal edge of the pecten (Fig. 1) and extends into the superior-temporal quadrant. Here, neuronal density reaches 24 000 mm^2 in the ganglion cell layer (GCL), compared to 4000 mm^{-2} in the periphery [84]. This specialisation is scarcely present in the early embryonic retina. Between embryonic day 8 (E8) and 4 weeks after hatching the central/peripheral gradient in cell density increases from 1.5/1 to 4/1 in the GCL. In the chicken retina two areas with high ganglion cell densities have been described, but these areas remain afoveate [46]. The highest GC densities of 19 000–22 000 GC/mm^2 in the dorsal area centralis are functionally related to the high visual acuity of chicken. These densities correspond to the higher photoreceptor densities in these areas, and to the presence of rods and four types of cones containing red-, green-, blue- and violet-cone opsin [254]. Different avian species have variously developed foveas depending on eye sizes and ecological habits [96]. In contrast to the function of the area centralis, that of the area temporalis remains elusive.

Retinal neuroblasts leave the cell cycle in a specific order with respect to their prospective cell type: the first cells to become postmitotic are RGC, succeeded by the other types of retinal cells [262,266,267,366]. While migrating within the retinal neuroepithelium, RGC express growth associated protein GAP-43 [351] both within the ventricular surface and the vitreal limiting membrane.

One of the initial steps towards directed outgrowth of axons, is the transition of non-polarised to polarised cells, thus making the axons distinctive from the dendrites [292]. This polarisation seems to be influenced by a diverse set of glucosaminoglycans [27], although chondroitin sulphate proteoglycan seems not involved in initial outgrowth of ganglion cell axons [28,29]. However, quantitative appearance of chondroitin sulphate proteoglycans (PG-M/versican and PG-H/aggrecan) may regulate the process of fibre growth [377]. Among the various extracellular matrix (ECM)-molecules heparan sulphate proteoglycan or agrin [130,141], collagen XVIII [139], laminin-1 and merosin (laminin-2) [132,141] were found in the early basal membrane in the chick retina. Most of these components

were also found within the retinofugal compartments beyond the eye cup, that is the optic nerve and tract. The slow progress in defining the role of glycosaminoglycans is mainly due to the pivotal effects described for these molecules [190]. In addition to ECM-molecules, radial glial subcellular compartments seem to be differentially instructive on whether ganglion cells form an axon or a dendrite: by using a retinal cryosection model, Bauch et al. [11] showed that radial glial endfeet instruct the formation of axons, whereas glial somata instruct formation of dendrites.

But also calcium binding proteins are expressed in the early retina as paradigmatically mentioned for paralbumin [145].

One of the interesting questions dealing with intraretinal axonal growth and directionality was addressed by Stier and Schlosshauer [314] who analysed the role of radial glial cells and concluded that their endfeet are likely to be instructive to axons. The axonal versus dendritic outgrowth seems also affected by radial glial cells within discrete layers of the retina [11]. Light microscopy shows that from the 3rd embryonic day on, the first RGC segregate from the ventricular matrix layer and become situated near the inner limiting membrane where they round up and soon extend axonal processes [273]. An immunohistochemical investigation verified the sequence of RGC differentiation in the chick [363] supporting an earlier hypothesis [228] for the rat retina [249]. Thus, before nuclear translocation, the ganglion cell neuroblast extends an endfoot to either side of the retina and then migrates keeping contact in both directions. In this way these earliest neurons of the retina may not rely on directional information provided by Müller cells, as it was generally assumed. Blocking experiments with antibodies to the β-1 subunit of the integrin receptor significantly inhibited the migration of RGC, thus indicating that Beta-1 integrins play a role in neuroblast migration [40]. Watanabe and Raff [363] also showed that after detachment of the ventricular process, the axon is extended directly from the vitreal endfoot, which will later be retracted or eliminated autocatalytically.

The typical dendritic morphology develops much later, although central RGC possess dendritic protrusions at E4 (Fig. 3A). After E7, spine-like processes grow out to form the dendrites, which branch extensively between Hamburger-Hamilton (HH) stages HH-34 and HH-39 (E8 to E13) [249]. As revealed by 1,1′-dioctadecyl-3,3,3,′3′-tetramethyl-indocarbocyanine perchlorate (DiI) staining of fixed wholemounts, immature patterns of dendritic branching are present in RGC at E4 to E10 yet without clear stratification in sublayers of the inner plexiform layer (IPL). The clear stratification appears at E16 [162,350]. Coincident with the formation of the IPL, the RGC cytoplasm shifts to the dendritic pole. Ultrastructurally, Golgi zones elaborate and rough endoplasmic reticulum (rER) becomes organised into parallel arrays [161]. Little is known when diversification of RGC takes place, whether target-derived signals influence diversification, and how subtype-specific projection patterns are established. Thanos et al. [342] used DiI in formalin-fixed retinas to demonstrate the morphological diversity of RGC in the chick. It appeared that at least eight classes of cells can be identified on the basis of size, dendritic shape and pattern of ramification. This morphological diversification may not necessarily correlate to molecular markers as was examined with antibodies staining two chemically distinct subtypes of RGC [375].

The question of how and at which stages embryonic positional information is encoded in the retina and tectum is of crucial importance towards understanding the molecular mechanisms of retinotectal connectivity. Ablation experiments with the prospective temporal retinal primordium around stage HH-11 of development caused immigration of prospective nasal cells into the posterior halfprimordium and ultimately resulted in the formation of a mosaic specification of the temporal hemiretina [81]. This phenomenologically impressive mosaic pattern consisting of both nasal and temporal cells within the temporal retina pointed to an early determination of the cellular positional specificity. Indeed, analysis of the protein patterns along the anterior-posterior axis at stage 11 revealed a clear asymmetry in the expression of four visible proteins spots, with the most prominent of them, a 40-kDa protein, exclusively expressed within the prospective nasal retinal primordium [334].

Creation of a double-nasal or double temporal retina by transplanting hemiprimordia resulted, in accordance with these results, altered fibre outgrowth behaviour in the Bonhoeffer in vitro assay [80] (Fig. 6D–F). However, some degree of respecification is possible as shown by analysing fate-determining molecules like chicken brain factor-1 and 2 (CBF-1 and CBF-2) [232]. These transcriptional regulators belong to the *winged-helix* family and are expressed in a mutually exclusive manner in either the nasal or the temporal half of the retina [378]. Misexpression of each of these factors caused changes in the retinotectal projection showing that CBF-1 and CBF-2 control formation of the map. It is, however, not shown whether these factors are expressed in a graded manner in accordance with the prediction of the gradient-model of Gierer [113,114]. Using a cell line as expression system, Yamagata et al. [376] showed that retinal cells first begin to express CBF-1 or CBF-2 according to their topographic positions, generate cellular descendants, which maintain the expression of CBFs, and then regulate the naso-temporal gradient of Eph-A3 [376]. The role of ephrins in intraretinal guidance remains unclear, because recent experiments in the mouse retina showed a kinase-independent axon pathfinding [16]. An asymmetric expression of SOHO-1 which is highly expressed in the nasal retina and at low levels in the temporal retina also suggested a role in patterning the naso-temporal positional specificity [69]. On

the other hand, establishing of the dorso-ventral axis seems to be controlled by Pax-2 and/or dialdehyde-dededrogenase-2 (DHD-2). Enzymes that synthesise retinoic acid from retinaldehyde are also spatially segregated in the early eye vesicle of the chick [223] and may control responsiveness to neurotrophic factors like brain-derived neurotrophic factor (BDNF) [224]. During the early retinal development an asymmetric dorsal-to-ventral increasing gradient of retinaldehyde results in production of the all-trans retinoic acid (RA) isomer with highest concentration in the ventral primordium. Local measurements of retinoic acid production and degradation indicate a horizontal retinoic acid free zone in the middle-dorsal retina, reminiscent of the pattern that can be observed in retinoic acid reporter mice (Mey, unpublished observations). Yet, the exact interplay and the hierarchical regulatory cascades still remain to be understood given that an ever growing evidence suggests that finely tuned genes control the later growth and navigation of axons.

To facilitate understanding of how the chicken retinotectal system develops, some typical stages of retinotectal growth of axons are schematically shown in Figs. 2 and 3.

3.2. Intraretinal growth of ganglion cell axons

Whereas positional specification to become either a nasal or temporal ganglion cell seems to occur at HH-9 to HH-10 under the control of hierarchically organised factors, the mechanisms of growth cone formation and guidance still remain under investigation. Growth cones, and hence axons, are directly formed at the endfeet that contact the inner limiting membrane [363]. Subsequent retraction of the endfoot may mark the identity of this cell which now becomes a projection neuron. Halfter et al. [139] injected collagenase into the vitreous at E3 to E6 and disrupted the basal membrane (BM) causing the retraction of the endfeet, thus resulting in a number of abnormalities in the axonal navigation. This experiment supported the instructive role of the endfoot/BM-interaction for the initial growth cone formation. Intraretinal grafting of small pieces of premature retina also revealed that BM-associated cues exist within the retina [131]. Also antibodies to NT-3 disrupted normal development and pathfinding within the retina [22] indicating that neurotrophins play a crucial role as trophic molecules.

There is a clear centre-to-periphery gradient and a temporal to nasal asymmetry in the generation of axons. At stage 15 (E2 to E3), well before intraretinal connections are completed, and shortly after secondary invagination of the optic vesicle, the first ganglion cell axons appear in the so-called central retina, 150 μm dorsal to the entrance of the optic stalk [120,157,175,280]. This central-peripheral gradient is also demonstrated with antibodies to a precursor marker 1 (PM1) whose expression reflects the pattern of development [150]. From the very beginning, the axons are polarised and aligned towards the optic stalk and grow in this direction, although many of them traverse into wrong directions and meander substantially, before they find the correct direction (Fig. 3). This can be easily demonstrated by retrograde labelling with DiI from the optic stalk at E3. It appears that several axons within the area centralis are meandering before they follow the correct path towards the optic fissure (Fig. 3A). The question of how the polarisation is controlled has not yet been answered. However, this may not require particular control since first axons may follow the optic vesicle evagination in reverse direction. More and more axons are added to fascicles, which now constitute the optic fibre layer (OFL). The intraretinal growth cones are 'spearlike'-structures with directionally oriented filopodial protrusions almost exclusively targeted to the optic fissure [239]. Each axon seems to join and follow the nearest existing fibre, such that a funnelling effect in the direction of the optic stalk appears. Virtually no branching of axons is visible within the retina. At the beginning, fibres frequently meander and cross each other. Later they appear more orderly and parallel, possibly due to stretching, as the retinal tissue expands by a factor of ~4 between E3 and E6 [120,157,235].

Two sublayers of fascicles can be distinguished in the OFL, one deep group of fascicles adjacent to the GCL, and one superficial, vitreal layer. This pattern results from the fact that peripheral axons are added on the vitreal surface of already present central fascicles, which are larger and have more dorsal inclinations towards the optic fissure [157]. This implies that a first level of retinotopic arrangement occurs already within the retinal OFL. By using anterograde tracing with DiI, Thanos and Bonhoeffer [339] found all intraretinal growth cones to be localised in the vitreal aspect of the OFL. As a rule, almost all fibres are directed towards the optic fissure. Some exceptions were observed in young retinas cultured in vitro (HH-17). In these wholemounts misrouted axons were directed towards the opposite, peripheral direction [135,136]. In a recent study Halfter [133] showed that the basal lamina has a crucial role in maintaining the ordered growth of retinal axons, since disruption of the lamina with collagenase resulted in aberrant growth.

3.3. Molecular paving of the intraretinal path

The question remains, which instructive molecules are responsible for axonal guidance within the retina. Local cues may be present either on cells, especially on glial endfeet and pre-existing axons (when the growth of pioneer neurons is assumed), or they can be displayed in the extracellular matrix. Anatomically, growth cones of elongating retinal axons are positioned immediately adjacent to neuroepithelial endfeet. This is seen in the retina, where the relevance of Müller glia endfeet has experimentally been confirmed [135], in the optic nerve and tract [307], and on the optic tectum [125,352]. In the chiasm,

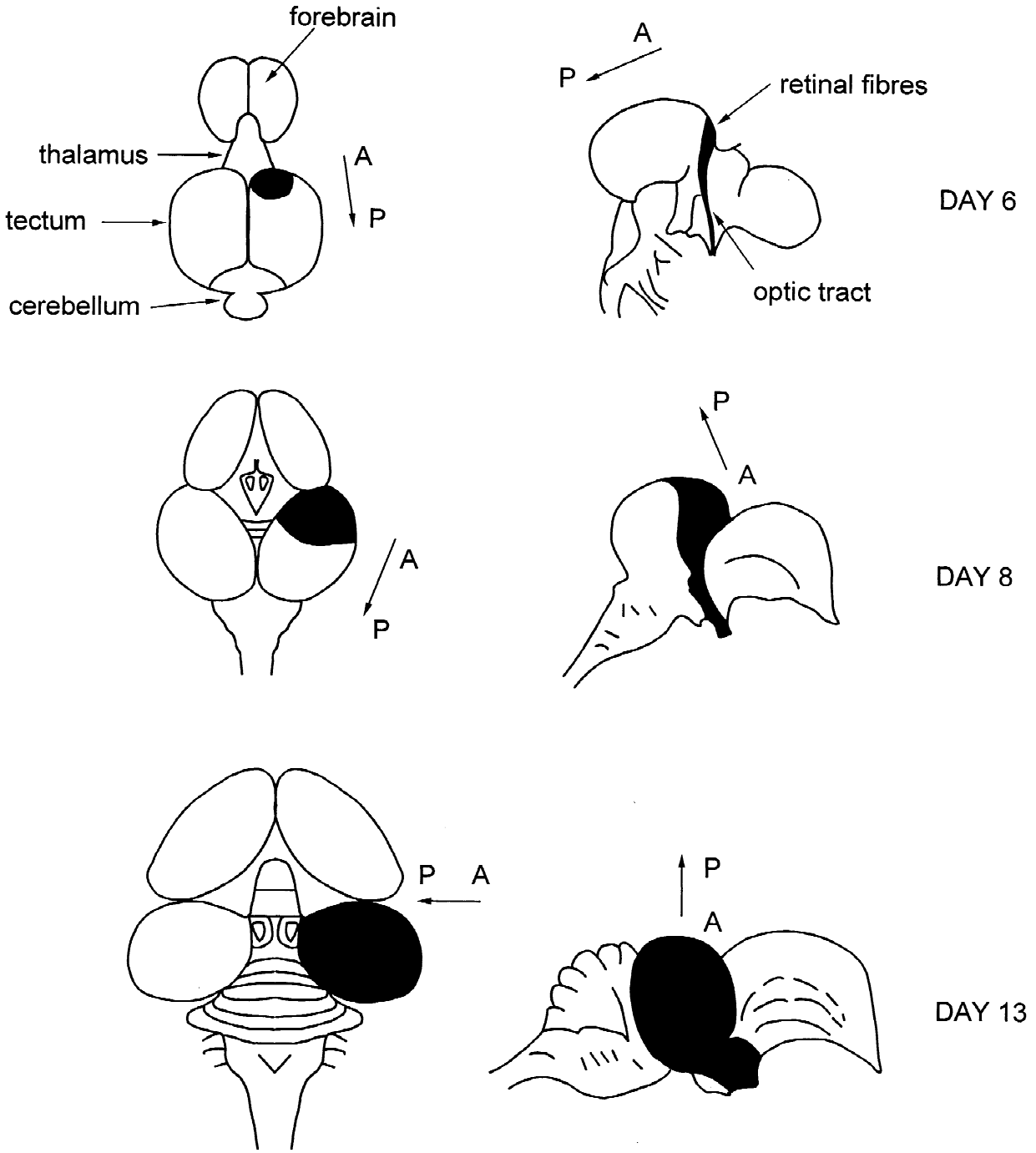
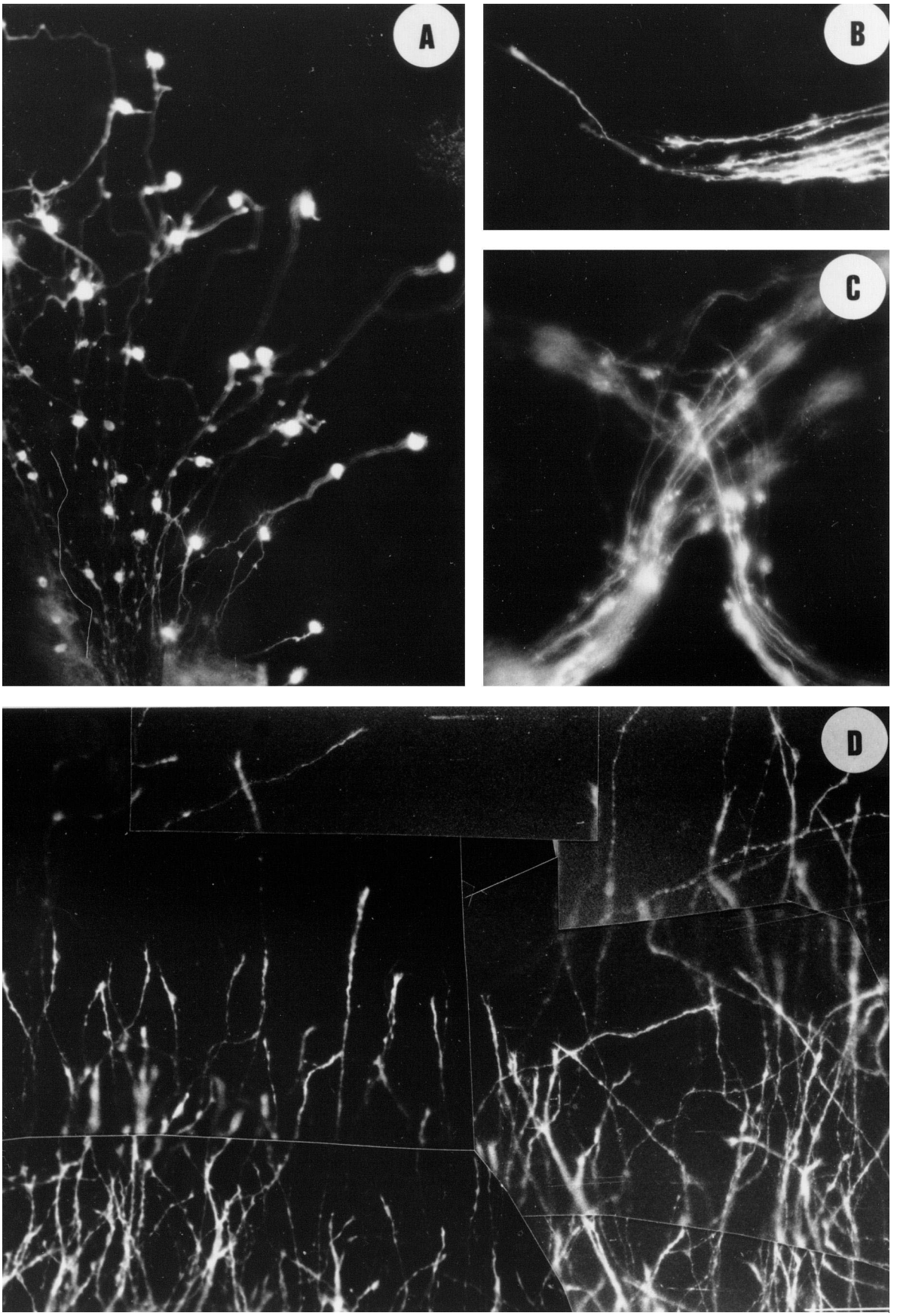


Fig. 2. Schematic drawings show the time course of the retinotectal innervation in coincidence with the anatomical growth and the topological transformations which take place between E6 and E13. Left column shows a dorsal view while right column shows a lateral view of the chicken embryo head at E6, E8 and E13. Arrows show the axial coordinates and the tectal rotation between E6 and E13. Black areas show advancement of retinal axons.

fibres re-orient in close contact with primitive glial cells [240]. Here we first discuss the putative molecules of guidance within the retina. Fig. 4 summarises the molecules proposed to be involved in guidance of retinotectal axons and the possible interactions between the axonal growth cones with their environment.

Fig. 3. (A) Retrograde staining of the earliest RGC within the area centralis at HH-23. Each spot represents an RGC whose axon had reached the optic chiasm midline at the time of fixation and retrograde stain with DiI. Note the irregular intraretinal routes of individual axons (arrows) and the onset of dendritic sprouts (arrowheads). (B) Anterograde staining of retinofugal axons within the optic stalk at HH-23. (C) Initial formation of the chiasm at HH-24 to HH-25 with intermingling axons from both optic nerves. Arrows show the midline. (D) Anterograde labelled axonal front on the tectal surface at E8 shows that some axons change their positions relative to others and meander over the tectum. Scale bars: 25 μm.


A
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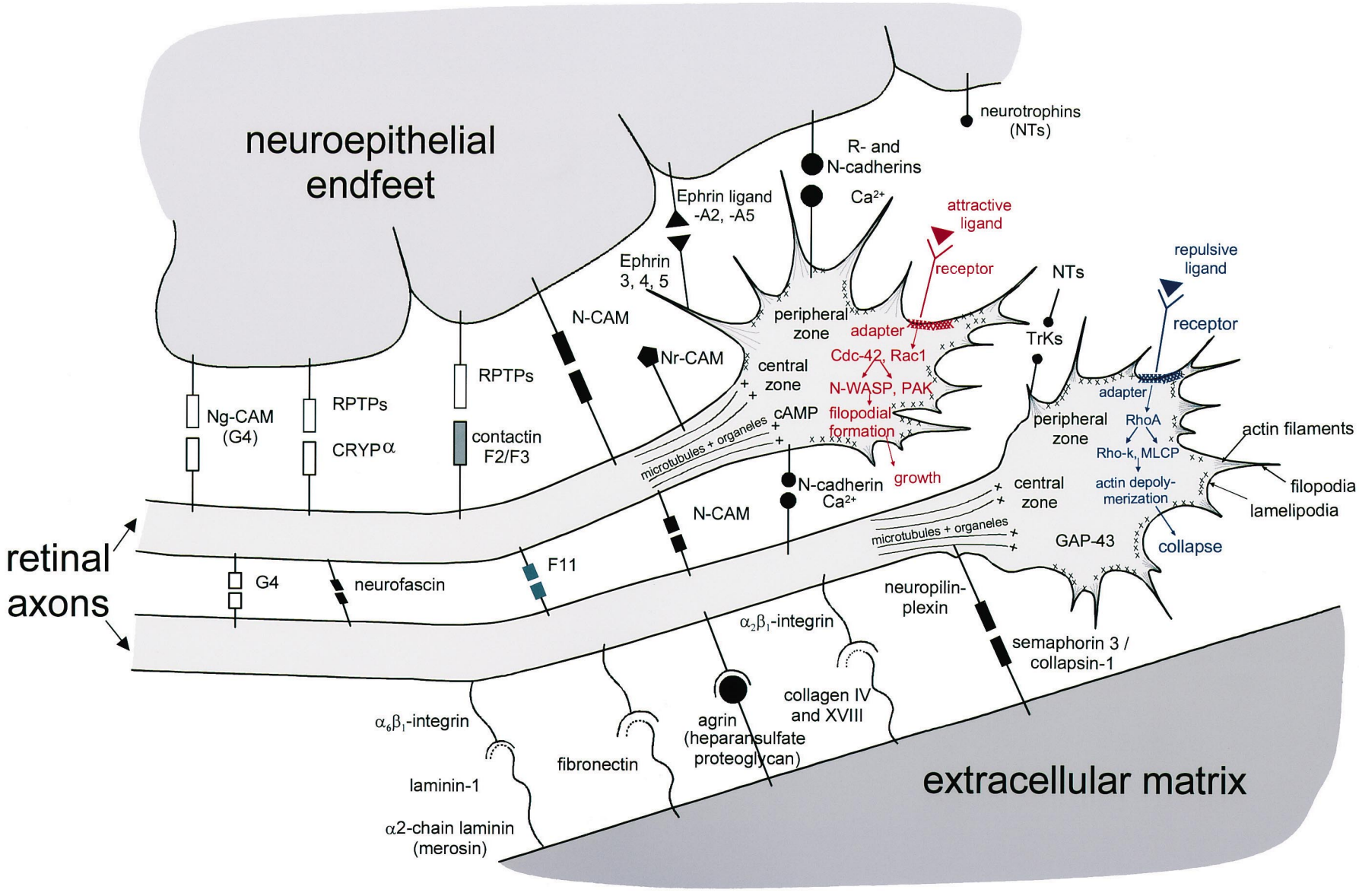

Fig. 4. Molecular decoration of the retinotectal growth cone and the major environmental structures including the extracellular matrix (ECM), the neuroepithelial and glial endfeet, and the neighbouring axons. One putative repulsive signal-transduction pathway (blue), and a chemoattracting pathway (red) are shown to demonstrate how external signals may be transmitted to filopodial extension or retraction. Abbreviations are explained in the Abbreviations list.

Intraretinal growth of axons is likely instructed and directed by interactions between the radial glial cells of Müller [72,314–316] until growth cones approach the glial endfeet at the vitreo-retinal border [135]. The basal lamina (or inner limiting membrane) seems to direct growth towards the optic fissure, as examined by Halfter and Schurer [137], who disrupted this membrane and observed chaotic fibre growth. In a similar fashion, interactions between growth cones and glial endfeet are assumed within the optic stalk and tract [307] and on the optic tectum [125,222,352]. At the molecular level growth towards the optic disc (fissure) requires expression of cell adhesion molecules (CAM) of the immunoglobulin superfamily like L1 and neural cell adhesion molecule (N-CAM) [27,28,30]. Intravitreal injection of antibodies to N-CAM at E3 (Fig. 6) disrupted the orderly formation of the fissure and resulted in massive overshooting of axons which failed to exit towards the optic nerve [337]. Injection of Fab fragments to L1 resulted in defasciculation of axons [115]. According to a demonstration [37], there is no doubt that abrupt changes in the molecular composition of the substrate is influential during axonal pathfinding. Some of the adhesion molecules like CD15 [4] show a spatiotemporal expression within the retina and are therefore associated with intraretinal axonal growth.

McAdams and McLoon [211] analysed the expression of chondroitin sulphate and keratan sulphate proteoglycans in the early retinofugal pathway and identified that the proteoglycan form of collagen IX was observed only in the retina at E2.5. Chondroitin sulphate was observed along the total retinofugal pathway. Other proteoglycans that are also developmentally regulated and constitute the basal lamina of the retina. Ring et al. [286] identified with the 9BA12 antibody a chondroitin sulphate proteoglycan whose expression coincides with onset and cessation of axonal growth, but seems not involved in promotion of axonal growth in retinal cultures [29]. In contrast to the mammalian retina, the chicken retina has myelinated retinal axons within the OFL [237]. The oligodendroblasts of the GCL differentiate to oligodendrocytes at E14, that is after axons have left the eye. In contrast to Müller cells, myelinating glia does not seem to influence axonal growth [237].

The retinal basal membrane also contains ligands for receptor tyrosine phosphatases (RPTPs) like the CRYPα that was recently shown to be involved in intraretinal axon growth [194,195]. Antibodies against CRYPα reduced axon growth on the basal membrane and changed the growth cone morphology acting on their filopodia. The data point to a balance between growth promoting and permitting molecules on one side, and growth inhibiting influences [316] on the other side. RPTPs are therefore ascribed a crucial role in the maintenance of intra-growth cone phosphotyrosine levels [195]. So far, the Müller endfeet in the BM seem to contain the putative transmembrane ligand CRYPα. Using immunohistochemistry and in situ hybridisation Ledig et al. [195] showed that distinct subtypes of RPTPs (CRYPα, CRYP-2 and PTPμ) are expressed along the retinotectal pathway in a spatiotemporal pattern that reflects development between E6 and E14 (Fig. 4). Yet, their function in vivo has not yet been elucidated. Since blocking of β1 integrin receptors results in massive reduction of outgrowth, growth cones may use these receptors to integrate growth signals from glial endfeet [290]. Using fluorescent antibodies and chromophore-assisted laser inactivation Pollerberg and coworkers inactivated the phosphorylated form of microtubule-associated protein MAP-1B in growth cones from chick retinal ganglion cells. In growing axons phosphorylated MAP-1B is concentrated in the distal axon and in the neck of the growth cone where it appears to stabilise the association of microtubules. Local inactivation of MAP-1B on one side induced a paralysis and retraction of the treated half of the growth cone, thereby causing a turn of the axon to the contralateral side [204].

Among the multiple factors that may guide axons within the retina, the fibroblast growth factor receptor seems crucial [30]. Also Netrin-1 and its receptors of the DCC-family seem to mediate axonal guidance at the optic disc, since loss of their function results in hypoplastic optic nerves [68]. Netrin-1 and netrin-2 are chicken-derived homologues of UNC-6, a laminin-related protein of *Caenorabditis elegans* [303]. The netrins comprise a small conserved family of cues, whose role in axonal guidance was first demonstrated in the floor-plate and later in the PNS (Section 4.3) [50,362]. It has not yet been shown whether chicken netrins are involved in retinotectal guidance.

It is widely accepted after three decades of research on adhesion molecules that chick retinal axons show indeed a preferential adhesion on some substrates such as chick L-1, poly-L-lysine, laminin and N-cadherin [35,196]. One has to distinguish between permissive influences provided by these molecules and instructive influences. The latter are assumed to determine the choice of pathways by providing positional and/or directional information. Permissive influences may be subsidised by additional biochemical or mechanical barriers [359,350,309]. It is not excluded that permissiveness may be enhanced by the moving growth cones which release proteases [70], especially at places which are densely packed by glioblasts like the supraoptic zone [240]. In the next chapter we first discuss the anatomical development of the optic pathway behind the optic nerve head, and then again introduce molecular candidates that may be involved in growth cone navigation.

3.4. Formation of the optic nerve

After embryonic day 3, the first fibres leave the eyeball and grow along the optic stalk (Fig. 3B). Anterograde staining in fixed embryos at HH-20 to H-24 revealed

individual axons with remarkably large growth cones. Typically one or two 'leading' axons are much larger than the secondary and tertiary waves of axons, each of them consisting of five to ten axons. The first axons arrive at the chiasm at HH-22 (Fig. 3C), and between HH-22 and HH-25 they advance through the contralateral optic tract (Fig. 3D) towards the tectum at whose anterior pole they arrive at E6 [129,336]. Earliest axons enter the stalk at its ventral side, and new fibres are added peripherally and ventrally such that the circular area occupied by the first RGC is transformed into a ventral crescent in the optic nerve [239,275]. Parallel to fibre invasion, the development of glioblasts proceeds in retino-diencephalic direction in the optic nerve. Axons elongate through the extracellular space, which is organised by a framework of radial glial processes from cells in the ventral wall of the stalk [239,277]. Glioblasts then divide and migrate radially, thereby separating optic fibre fascicles. The marginal fibre growth is further supported by glial cell death in the ventral wall [239–241]. This may be induced by enzymes secreted from advancing axonal growth cones. Some of the ECM-proteoglycans may be produced by the growing axons, as revealed by immunostaining to chondroitin sulphate [211]. Applying anterograde staining with rhodamin-B-isothiocyanate (RITC), Thanos and Bonhoeffer [336] demonstrated a separation of fibres according to their retinal origin in the optic nerve and tract. Ventral retinal fibres grow dorsomedially, whereas dorsal fibres lie ventrolaterally in the optic pathway. Also the central to peripheral order is maintained in a similar pattern as in the OFL. Before crossing to the contralateral side in the optic chiasm, nasal fibres occupy the medial, and temporal fibres the lateral half of the nerve. In the optic tract, this order no longer prevails: nasal and temporal fibres lose their original neighbourhood relationship and become mixed [86,235,336]. In the chick, fibre crossing in the chiasm is complete, though a transient ipsilateral projection appears during embryogenesis, which is soon eliminated [252,337,368]. The optic nerve head itself is retinotopically represented in central projections as they locally lack retinal fibres [271].

In the first electronmicroscopic examination of the optic nerve in chick embryos, Rager [273] described its development between E8 and 3 months after hatching. A continuous increase in cross-sectional area from 0.15 mm^2 to ~2.3 mm^2 was observed due mostly to glial proliferation and myelination of axons. Around E15 oligodendroglial processes multiply and start to envelop thicker axons. At E18, 3% of them are myelinated. Most of the myelination takes place during the early postnatal period, but continues to add to number and size of myelinated fibres [6,7]. Axonal diameters increase in proportion to the myelin sheath, thus providing for optimal transmission parameters [273]. In physiological experiments with electrical stimulations of the optic nerve head in the retina Rager simultaneously recorded from the tectal surface of chick embryos. The youngest embryos with successful recordings were 8 days of age. Afterwards, changes in electrical properties (reduction in latency, increase in amplitude) paralleled the ultrastructural maturation [273,274].

3.5. Development of the optic chiasm

The importance of the optic chiasm in determining retinotopy was recognised by Ehrlich [85]. As mentioned, the first few axons approach the chiasm at HH-20 to HH-21 to cross with axons from the contralateral retina at HH-22 to HH-24 and later (Fig. 3C). Histologically, the chiasm is well-demarcated from the supra-optic commissure and from the olfactory pathway at HH-26 [306,308]. At the midline, a 'chi-like' crossing can be observed with anterograde tracers (Fig. 3C) and details of the growth morphology can be examined (Fig. 3C). Drenhaus and Rager [75,76] used qualitative and quantitative microscopy to show that alternating tiers (sheets) of bundles interdigitate between E4 and E19. There were ~32 tiers at E18/19. Maturation of the chiasm follows a gradient from ventral to dorsal, with the last growth cones to arrive at E18, thereby reflecting the spatiotemporal retinal development. It is apparent that the tiers represent fibres of either retina, thus suggesting a degree of asymmetric chiasm development. Indeed, anterograde staining of fibres from both developing retinas at HH-20 to HH-22 revealed that there is an asymmetry in the arrival of axons. In more than 90% of all embryos axons from the right retina are more advanced than their counterparts from the left retina. This asymmetry may account for the misrouting of some axons to form either the transient ipsilateral retinotectal pathway [252,337,368] or the transient small retino-retinal pathway [129,335]. Rarely, some axons grow rostrally into the olfactory region of the telencephalon as observed in the mouse [308]. A number of topological transformations occur along the retinotectal pathway, including the chiasm. Rager et al. [279] demonstrated with local injections of horseradish peroxidase (HRP) into the tectum and analysis of retrogradely labelled axons that the first of these transformations occurs within the optic nerve head, and a second behind the chiasm to form the optic tract.

Glial cells in the optic chiasm arise from suboptic centres of the diencephalon floor. Before any axons arrive at the midline (HH-20) a spongy tissue with dying cells is observed in the suboptic centres. This area was named 'knot' in the mouse [306,307] and is characterised by cataclysmic cell death at pre-axonal stages. Clusters of primitive glial cells replace the necrotic zone in the suboptic area at HH-23, to move medially and converge at the midline at HH-27 [238]. The prechiasmatic area of degeneration appears just rostral to the chiasm at HH-25 to HH-26 [239,240]. The topological association of the primitive glial cells with incoming axons that change their direction from lateromedial to rostro-caudal is obvious, yet not causal. The usually studied glial markers vimentin and

glial fibrillary acidic protein (GFAP) are also expressed in the chiasm glial cells, as shown recently in a systematic study of the time between hatching and adulthood [310]. Wizenmann et al. [372] showed that, in contrast to ipsilaterally projecting rat retinal axons, chicken axons pass through chiasm membranes without being repulsed.

In contrast to its absence on neurites and glial cells, laminin-1 was found at the endfeet of neuroepithelial cells along the outer margin of the optic pathway at early development (Fig. 4). In contrast to alpha-1, beta-1 and gamma-1 chains of laminin-1, laminin alpha-2 chain (formerly laminin M chain) is expressed in the optic nerve and optic chiasm [229]. Also a number of cell adhesion molecules are expressed in the chiasm (discussed below) and are putative candidates for influencing chiasm development [278]. Ephrin ligands seem to be candidates for axonal pathfinding through the chiasm, because overexpression of Ephrin-A2 and -A5 resulted in pathfinding errors with increased and stable ipsilateral projection [82].

3.6. Axonal trajectory within the optic tract

The optic tract extends between the chiasm and optic tectum and represents a flattened structure in the diencephalon. First axons arrive at the optic tract at HH-22 (Fig. 2D). The course of axons within the optic tract is considered retinotopic with the ventral fibres lying laterally [75,76,86,279,336]. First produced axons in the retina form the deeper part of the optic tract whereas peripheral retinal axons are added on top of pre-existing axons in a 'sheet'-like fashion. In accordance with Rager et al. [279] a second topographic transformation occurs in the optic tract with a clockwise rotation of 90° and flattening to a band. This may reflect the developmental rotation of the tectum by 90° between E6 and E9 (Fig. 1). Some axons bifurcate along the optic nerve to innervate underlying thalamic nuclei [352]. A prominent bundle of axons diverges from the optic tract to traject along the mesencephalic midline and terminates within the prospective isthmo-optic areas at E6. This bundle is transient between E6 and E12 and associated with the projection of isthmo-optic axons to the retina [251]. Repellent molecules like collapsins are assumed to inhibit branching or invasion of the diencephalon by axons, thus steering them to their major target, the tectum [349]. Whether guiding pioneer neurons and fasciculation of axons with the same destination constitute a general morphogenetic principle in vertebrates is still controversial [70]. After observation of fibre growth in the tectum Goldberg [119] and Thanos and Bonhoeffer [336] argue in favour (Fig. 3D), whereas Harris [148] denies the existence of pioneer neurons (in *Xenopus*).

3.7. Molecular constituents of the retinofugal pathway

3.7.1. Extracellular matrix (Fig. 4)

The exit of axons from the retina is associated with changes in the molecular composition along the environment, initially made up of ECM and immature neuroepithelium, by the influence of neurotrophins [353] and later by addition of axons and maturing glial cells [361]. Such changes seem to trigger secondary events like spontaneous activity [374] transient axonal branching or downregulation of cell-adhesion molecules. In vitro, a developmental reduction of the response to different ECM-molecules [52,53] is observed in retinal ganglion cells, especially, as mentioned, the adhesiveness to laminin [54,55] and also to fibronectin [142]. Corresponding changes in the profile of proteins precipitated by a 'cell substratum attachment antibody' [143] and of a 'neurite outgrowth factor'-receptor [329] have been reported. Most remarkably, the expression and the functional status of integrins change during development. Investigations by de Curtis et al. revealed molecular causes for the declining RGC-response to laminin: between E6 and E12 the content of α_6-mRNA and -protein is reduced to ~20%, provided the ganglion cells made contact with the optic tectum [63,64]. In neurons that fail to innervate the target, the $\alpha 6\beta 1$ integrin and several others continue to be expressed, but still loose their activity. Investigations on the developmental regulation of integrin subunits in general show that different α-subunits are expressed at different times, whereas β-subunit expression appears more uniform, both spatially and temporally [32]. A specific antibody against the $\beta 1$-subunit, 'TASC', restores the ability of RGC-axons to grow on laminin and collagen IV after they have lost the responsiveness. Additionally, it reduces cell adhesion to vitronectin. This is taken as evidence that TASC-binding reverses a posttranslational downregulation of laminin-binding that may be triggered by synaptogenesis or cell–cell-contact [242]. The interactions between the developing RGC and collagen are mediated by $\alpha 2/\beta 1$ integrin, which, however does not mediate axonal extension [23]. Expression of chimeric chicken/Xenopus $\beta 1$ integrin after injection into the eye primordia resulted in reduction of process outgrowth in vivo, but unaltered pathfinding of the axons [198].

3.7.2. Adhesion molecules (Fig. 4)

The first category comprises glycoproteins of the immunoglobulin (IG) superfamily characterised by immunoglobulin domains and fibronectin type III repeats. They mediate Ca^{2+}-independent homophilic binding and heterophilic binding to heparin. Axons of developing RGC are known to express N-CAM, which is also present on marginal endfeet of neuroepithelial cells along their presumptive route [307]. Intraocular injection of antibodies to N-CAM (at E3.5) disrupted axons in the optic stalk, but not in the retina [307]. Such antibodies also prevented the orderly fasciculation of fibres at the optic fissure and throughout the pathway to the tectum [341]. In this experiment, a number of axons occupied ectopic positions on the tectal surface later, but the general direction of the

majority of fibres was not disturbed. Thus, it is unlikely that N-CAM distribution provides relevant information for marking the visual pathway. The authors suggest that the correct path of later appearing axons may depend on their ability to follow pre-existing fibres by selective fasciculation [341]. This had previously been proposed by Arees and DeLong [7], who demonstrated temporary junctions between developing retinal axons in the chick embryo. Several other cell surface glycoproteins, particularly neurofascin [282], G4 (neuroglial cell adhesion molecule, Ng-CAM), and F11 [45] seem to mediate retinal fibre fasciculation, as has been demonstrated with antibodies interfering with axon–axon interactions in vitro. Results obtained with chicken retinal explants in culture show that fibre fasciculation is sensitive to cytoplasmic protein phosphorylation, leaving levels of CAM expression unaffected [44]. Axonin like 1 (A-L1) and Ng-CAM are expressed in strong coincidence with the formation of the retinotectal pathway, whereas older axons lose the A-L1. This led Rager et al. [278] to conclude that A-L1 is a marker for newly formed axons, and plays a role in the organisation of the pathway. Other developmental modifications of RGC include decreased polysialation of N-CAM and decline of N-cadherin expression [42]. It is unknown whether these regulations serve to prevent overshooting of fibres, reflect adaptations of growth cones to a changing environment as different guidance cues are sequentially encountered, or have still other causes.

Further cell-adhesion molecules of the Ig-superfamily have been demonstrated in the chick visual system: Ng-CAM [38] and Nr-CAM [127] share 40% of their amino acids and are prevalent during retinal development. Antibodies against another immunoglobulin cell-adhesion molecule, 'BRAVO', specifically stain fibres in the retina, while Ng-CAM is present uniformly in the optic pathway [65]. Onset of axonogenesis in the retina is also accompanied by expression of SC1/DM-GRASP [263]. In a modified explant system Morales et al. [227] showed that Bravo/Nr-CAM and G4/Ng-CAM display synergistic effects on axonal growth. Functional roles of these molecules still remain to be proven.

3.7.3. Cadherins (Fig. 4)

The second category of adhesion molecules comprises cadherins which have one highly conserved cytoplasmic and four extracellular domains, the former associated with catenins, which interact with cytoskeletal elements. Cadherins mediate Ca^{2+}-dependent, homophilic binding [126]. R-cadherin is expressed in chick retinal neurons and glial cells mainly during later embryonic life and post-hatching [167,328]. N-cadherin, which is 74% identical to R-cadherin, has been detected on growing retinal neurites, but since — like N-CAM — this molecule is unspecifically expressed in most tissues of the embryonic chick its role as directional guidance cue is dubious [70]. Both cadherins are expressed within the chicken retina and optic nerve [283,284,373]. The levels of cadherin expression are controlled by an endogenous protease. Insulin can downregulate N-cadherin in the chick retina [288]. The importance of N-cadherin has been recently documented by García-Castro et al. [109] who showed that the molecule is involved in establishing the body's midline-asymmetry. However, there is no evidence for a function of this molecule in establishing the central nervous system (CNS) midline of optic chiasm. The expression of R-cadherin in the optic stalk displays a complementary pattern compared to that of N-cadherin. A number of different cadherins are associated with specific projections in the avian visual system. For instance, cadherin-6B and cadherin-7 are found in displaced RGC and the accessory optic system; R-cadherin, cadherin-A, cadherin-B occur in different tectal neurons. They are expressed by neurites during the time of axon elongation and restricted to subsets of growing fibre tracts [283]. Therefore, a role in retinal fibre guidance is being discussed, but experimental proof is lacking so far [17,283]. Since a number of nervous system cadherins have been cloned [325], the Ca^{2+}-dependent system of cell surface interactions may turn out to be far more complex than anticipated. Klostermann and Bonhoeffer [183] showed in the stripe assay that Ca^{2+}-dependent molecules are involved in axonal growth, but not in specific signalling associated with choice of a pathway.

3.7.4. Integrins (Fig. 4)

The third category comprises integrins which are heterodimers consisting of one α- and one β-subunit. They normally mediate binding to ECM-molecules. A variety of ECM-components [41] facilitates neurite growth in vivo. Laminin turned out to be most powerful with respect to retinal axons; functional but less effective growth substrates are collagen IV and fibronectin [142]. Other ECM-glycoproteins like vitronectin and thrombospondin [243], and possible receptors [73,329] are being investigated. One obstacle to real guidance functions of these molecules is that retinal fibres seem not to have contact with the basement membrane [207]. Therefore, most of these ECM-substances may be irrelevant. Exceptions are the isoforms of laminin, which also occur on cellular membranes. The best candidate, laminin-1, is also the most thoroughly studied one. Cohen and his collaborators demonstrated that laminin-1 is transiently expressed on neuroepithelial endfeet along the prospective pathway of retinal fibres. This happens during the 1st week of chick embryonic development, but not later when RGC axons have reached their target [52,54]. It is reported that in vitro, axons lose their responsiveness to the neuritogenic effect of laminin at the same time [55], although, as Cohen's group discovered later [54], the laminin–fibronectin receptor complex remains present throughout embryonic development. The α6β1 integrin has been sequenced and identified as the functional laminin receptor of developing chick retinal cells [63,64]. However, even the role of laminin must be

qualified by admitting that its distribution is too unspecific to guide retinal axons directly to the tectum [149,156]. At least, it appears to be largely responsible for the growth promoting property of the pathway selected for other reasons. Laminin and all other substances tested in vitro, are permissive rather than instructive molecules. However, graded distribution of guidance cues like Ephrin-A2 interferes with activation of integrin β-1 [159], and perhaps with the ability of growth cones to move on laminin.

3.7.5. Repellent molecules (Fig. 4)

A fourth category comprises repellent molecules first recognised in vitro and assumed to be involved in axonal guidance by inhibitory interactions [358]. When investigating interactions between axons of different origin, Kapfhammer and Raper [177] found that growth cones distinguish between subsets of neurons. For example, after contact with ciliary neurites, retinal axons react with collapse of their growth cone. The axonal tip also detaches from the substrate and growth cone motility is inhibited. Avoidance reactions which can alter the direction of axonal growth may be induced by inhibitory molecules in vivo [59,358]. The idea has primarily been employed to explain specific fibre guidance in the tectum, which will be discussed in Section 4.2 in more detail. Yet, repellent molecules may also provide a key to understanding the growth of longer fibre tracts. At decisive positions along the pathway, chemical barriers would then channel the advancement of fibres. This has been shown to be the case for keratan sulphate present in the glial roof plate of spinal cord and optic tectum [309]. Repulsive properties for several tenascin-related molecules appeared in various in vitro assays [91]. More generally, it is assumed that astrocytes aid in the formation of neural structures, especially by defining repulsive territories for elongating CNS axons. Extracellular matrix components including cytotactin and J1/tenascin are discussed as mediators of this morphogenetic interaction [91]. So far, however, neither have any receptors for repellent molecules been characterised nor do we understand the process of their intracellular signal transduction [181]. Although no particular second messenger has been identified in this context, concepts concerning the role of Ca^{2+} in growth cone behaviour gain in importance [170,180,191]. However, this stands in contrast to experiments of Klostermann and Bonhoeffer [183] who showed that blocking of Ca^{2+}-dependent pathways changes the general axonal growth but not the specific behaviour imposed by repellent signals. Loschinger et al. [202] used the stripe assay and time-lapse cinematography to conclude that the retinal growth cones respond to different environmental cues by using different second-messenger systems.

Repellent signalling to axonal growth was also observed with the Slit-family, first described in the midline of *Drosophila* [33,171]. The ubiquitous appearance of Slit-molecules was shown in the rat retinofugal axons where the Slits control pathfinding by preventing them from invading non-target diencephalic tissues, thus funnelling them toward the midbrain [287]. By looking at the expression patterns of Slit proteins in *Xenopus*, Cheng et al. [49] concluded that these conserved chemorepulsive molecules are involved in axonal guidance in multiple regions of the embryo. In rodents, Slit and Roundabout (Robo) are expressed in complementary patterns in the developing forebrain and various areas of the CNS [245].

3.7.6. Semaphorins and collapsins (Fig. 4)

These molecules which were first been described in insects [50,186] are putative secreted proteins similar to chicken collapsin [187,203]. Collapsin-1 is a glycoprotein of 100 kDa with no transmembrane domain, and a highly basic region near its C-terminal sequence [203]. The family of collapsin-like molecules act as repellent cues, are developmentally regulated and seem to mediate growth cone rearrangements by modulating Rac-1-triggered actin depolymerization [173]. Raper's group proposed that semaphorins bind neuropilin with differentially expressed receptor components [93], and interact with the receptors through one binding site that mediates the biological response and one site that potentiates this response [187]. The role of semaphorins in guiding retinotectal axons remains elusive. Takahashi et al. [326] studied the neuronal and non-neuronal forms of collapsin-1 binding sites in the chick and concluded that they differ from semaphorin binding sites. Semaphorins A and E antagonise neuropilin-1 and agonise neuropilin-2 receptors [327]. Fournier et al. [98] showed that semaphorin 3A enhanced the endocytosis at sites of F-actin co-localisation during the process of growth cone collapse. There is increasing evidence that semaphorins are suitable tools to dissect specific growth cone signalling cascades due to their specialised action on subsets of receptors.

3.8. Growth of axons over the tectum and termination within the synaptic layers

Being the most prominent anatomical and functional structure of the avian brain, the tectum and its cytoarchitecture has been extensively investigated [269,221,270,271]. However, its multi-layered organisation and the high number of neuronal types also attracted the interest of developmental biologists, and since the 1990s increasing attention of molecular biologists (for further references, see Ref. [221]).

First retinal fibres arrive at E6 (Fig. 2), when maximal cell proliferation occurs within the tectum. The intratectal differentiation is most advanced within its rostral and ventro-lateral portion and less developed in the caudal and dorso-medial parts [58,192,193]. This temporal-spatial gradient of development matches the sequence of arrival and termination of axons from the retina. Axons arising from the retinal area centralis terminate within the tectal

area centralis at around E8 to E9 and further axons from more peripheral retinal areas are added in an oval pattern around the area centralis, thereby keeping a rough retinotopic arrangement. The superficial coverage and formation of the OFL is completed at E12 to E13 (Fig. 2), followed by a similar gradient of tectal innervation between E12 and E14 within the stratum griseum et fibrosum superficiale (of the tectum) (SGFS) [193,192,301,352]. The SGFS consists of five cellular (a, c, e, g, i) and five plexiform sublayers (b, d, f, h, j). The latter contain dendritic processes from the SGFS neurons, and from deeper tectal neurons together with retinal arbours which terminate at different depths (Figs. 3C and 8). Yamagata and Sanes [375] studied the target-independent diversification and the target-specific projection RGC that are immunoreactive for substance P and ACh-R β2 subunit. They concluded that RGC and their tectal targets can chemically differentiate in each others absence. This reflects the presence of complementary cues that are responsible for lamina-specific connectivity.

There is controversy about the appearance and location of the first retinotectal connections. McLoon [219] has detected synapses of (previously HRP-labelled) retinal fibres in the anterior tectum even earlier, namely at E7. Most authors, however, report that the first retinotectal synapses do not occur before E11 [41,274,257]. This contradiction gains relevance in the light of the discussion about the way how the correct retinotectal connections are established. This projection obeys a topographic order whereby the first arriving axons, which belong to central RGC in the retina, will be connected to the (somewhat rostroventral) area centralis of the tectum. For this reason, the question of where the first fibres leave the SO and form synapses in the SGFS has aroused considerable interest. Based on intraocular [^{3}H]prolin injection and autoradiographic detection of anterogradely transported proteins, Crossland and collaborators [60] localised a restricted, oval area near the centre of the tectum, where at E10 retinal fibres first submerge into the SGFS. Concurrent with the tectal cytoarchitectonic differentiation, this area expands concentrically as fibres from more and more peripheral parts of the retina are added [60,61]. Experiments involving early enucleation of the optic cup indicate that the mitotic patterns and the initial differentiation of tectal neuroblasts are independent from retinal axons [57,58]. Retinal and tectal spatiotemporal patterns of development seem to be matched like two independent clockworks. The central tectal area, which is due to receive afferents from first central RGC is also the first region to become receptive for axons [60,275,339]. These findings are contradicted by McLoon [219], who, as mentioned, reports to have seen synapses already at E7 in the anterior tectum. If the earliest axons immediately penetrate the rostral SGFS and form synapses, these contacts must be released subsequently, because (a) the axons will project to the central tectum in the adult chicken, and (b) by E8 the horizontal growth of the rostral tectum is completed. McLoon, therefore, postulated shifting connections, similar to the situation in frogs and fish. His claims, however, oppose all other investigations, which show that the first retinal fibres proceed over some distance of unoccupied tectal territory before reaching their destination where they form terminal arbours. This observation gains central importance for Rager's order-in-time→order-in-space model, which explains that the first fibres simply make contacts where they encounter a receptive, mature tectal area [275,279]. These initial contacts may then be transformed into synapses [274,257]. So far, no further evidence for shifting connection in the chick tectum has been obtained.

3.9. Refinement of the projection by apoptosis and fibre death

Neuronal cell death in the chick retina occurs mainly between E12 and E16 and accounts for the elimination of at least 20% of the RGC. The onset of normal cell degeneration concurs with cessation of mitosis at the ventricular side, and cell death seems to precede the phase of cytological maturation [162,163]. As we will discuss later, degenerating neurons are probably RGC that fail to establish central connections, and cell death acts to match retinal and central patterns of the developing visual system [276]. This matching is necessary, because retinal development proceeds independently from processes in the brain. The autonomy lasts until, after E12, retinotectal fibres form synaptic contacts and the survival of RGC requires successful connection with central targets. Ehrlich and Mills [87] suggested that the retinal terminals are self-absorbed. McLoon [218] suggested imprecision of the initial projection that becomes refined later. There is common agreement now that inappropriately projecting cells and fibres are eliminated.

Cytodifferentiation of retinal ganglion cells including formation of dendrites seems also to be intrinsically programmed. This can be inferred from experiments involving early removal of the tectal primordium, so depriving RGC of their targets [162,163,350]. In addition, these studies suggest that the temporal to nasal progress of retinal maturation follows endogenous rules, independent from the (corresponding) target development. Schlosshauer and co-workers have immunologically identified a protein that is associated to RGC axonal outgrowth in vivo, and downregulated after tectal innervation. But its expression is not influenced by optic nerve transection and, therefore, must be independently regulated [296]. Sargent [291,292] suggested that nicotinic acetylcholine receptors are involved in the distinction between axons and dendrites. Post-lesional plasticity of RGC dendrites [261] also support the notion that regulation of neuritic morphology is internal to the retina. In addition, the complete maturation of ganglion cells occurs after the period of cell death and

depends on central innervation [350]. Castagne and Clarke [43] induced ganglion cell death by axotomy and concluded that the time-course of this target-dependent death involves oxidative stress. Tectum-dependent survival was assessed at several levels. Garner et al. [111] analysed the TrkB isoform expression and postulated that BDNF acts as an anterograde and/or retrograde trophic support. Herzog and von Bartheld [151] and Herzog et al. [152] examined the BDNF-expression and concluded that this factor like FGF [128] supports survival of RGC. Fuhrmann et al. [106] found that the ciliary neurotrophic factor (CNTF)-receptor is expressed in all major neuronal classes in the chick retina, including RGC. Williams and McLoon [368] observed that the transient ipsilateral projection is not solely eliminated by cell death, but perhaps by elimination of collaterals. Injections of tetrodotoxin (TTX) that blocks and grayanotoxin I (GTX) that opens sodium channels, showed that both drugs interfered with the regression of overshooting fibres and arborizations outside the terminal zone [185]. In more recent studies, Ernst et al. [90] observed that nitric oxide (NO) synthase (NOS) is expressed by tectal cells. Both NO and *N*-methyl-D-aspartate (NMDA)-receptors are involved in refinement of the projection. The NO-mediated elimination of axons is abolished in presence of BDNF that protects from NO-mediated growth cone-collapse at local level of stabilising acting filaments [89,90]. It seems therefore likely that channel-gated neuronal activity plays a crucial role in refinement of the projection. The proportion of RGC and axons which die during development was estimated to be 35% in the chick, and therefore smaller than in mammals (54–74%) [74].

4. Experimental models to study the retinotectal topography

The basic idea of a chemical gradient that attracts axons was first pronounced by Ramon y Cajal [281]. After Sperry's formulation of the chemoaffinity hypothesis [312], based on his experiments with frog eyes, chemical coding became a key concept in developmental neurobiology. For the development of the retinotectal projection the chemoaffinity hypothesis proposed that molecular gradients ''with their roughly perpendicular axes superimposed on the retinal and tectal fields (cells) establish complementary relations in the affinity forces . . . linking corresponding points in the two fields'' [312]. Since then experimental work has been rewarded and paralleled by theoretical considerations. The extreme complexity we are faced with deserves dissection of the experimental data into in vitro data, in vivo experiments and theoretical concepts associated with either of the results.

4.1. The chick retinotectal projection in vitro and the evolution of the stripe assay

The clear formulation of the chemoaffinity hypothesis revived in vitro experiments introduced by Weiss [364] and elaborated by Letourneau [197] who showed that, in principle, axons growing in culture can distinguish between different substrates, and prefer to grow on poly-ornithine and collagen rather than on palladium-coated surfaces. As an extension of these findings, a variety of favourable substrates for retinal neurite outgrowth were tested subsequently. Components of the ECM-like laminin [10], fibronectin and collagen IV [142], the whole retinal inner basal lamina, matrix of retinal pigment layer [134,140,143], tectal membranes [42] and synthetic poly-L-lysine were found to permit retinal axon growth.

A decisive role in this growth and navigation in vitro is ascribed to the growth cone, located at the tip of all advancing axons and dendrites. This highly motile sensory apparatus mediates interactions with the environment through a number of receptors for neurotrophic factors and cell adhesion molecules [122,231,332]. Chicken retinal ganglion cell growth cones express receptors to laminin ($\alpha_6\beta1$ integrins), collagen IV, fibronectin, N-CAM, Nr-CAM, G4-neurofascin, N-cadherin, R-cadherin, F11 and others [222]. The actin filament containing filopodia are responsible for adhesion to the substrate, whereas the microtubules containing lamellipodia are rather membranous protrusions specialised for orientation [259]. The knowledge of the growth cone organisation and molecular decoration has been addressed in recent reviews [122,231]. The major molecules found in retinotectal growth cones, and interactions with the environment are shown in Fig. 4.

In parallel to the growth cone work and to the studies of retinal growth-substrate interactions, the group around Bonhoeffer have asked whether retinal axons recapitulate specific, and topography-relevant, features in vitro. By using a Y-like system of retinal explants of temporal origin Bonhoeffer and Huf [21] identified that growth cones from the temporal retina grew preferentially along temporal axons, whereas growth cones from the nasal retina did not distinguish between nasal and temporal axons. In a co-culture system of retinal explants and tectal membranes of either anterior or posterior tectal origin, the same investigators showed that temporal axons preferred to grow on anterior tectal membranes whereas nasal axons do not [19,20]. Halfter et al. [138] identified in a similar assay of neurite/membrane confrontation a strong anterior-posterior polarity along the retina with posterior tectal membranes adhering on nasal retinal axons. These results demonstrated the existence of directional cues in the tectum, compatible with target-derived cues [18]. Contemporary mathematical models based on the complex neurite-pathway interactions [113] revealed compatibility between the experimental data and the differential adhesion-based chemoaffinity. In a modification of the retinotectal in vitro assay temporal retinal axons were shown to be sensitive to the slope of a gradient from posterior tectal membranes [9]. So far, the data are compatible with chemoaffinity hypothesis, although the instructive molecules involved in this process remained to be shown.

The cell signalling pathways for growth cone repulsion were studied in a modified stripe assay combined with a 'Campenot chamber' that allowed examination of the effects of several drugs interfering with cell signalling [183]. They ruled out the need of Ca^{2+}-dependent adhesion molecules like cadherins, and implied the involvement of calmodulin and protein kinase C in growth cone elongation but not a 'choice' between alternative substrates. They concluded that parallel signalling pathways take part in guidance of axons in vitro. How finely the graded activities are tuned in the tectum was demonstrated by von Boxberg et al. [354]. They used Bonhoeffer's stripe assay and showed that a specific fraction of the tectal membranes also caused substrate selection by nasal retinal axons. This additional tectal activity remained uninfluenced by ephrin ligands indicating repulsion-independent mechanisms of guidance. The later experiments of Hornberger et al. [158] confirmed these results by showing a sensitivity of nasal axons to phosphatidyl inositol-phospholipase (PI-PLP)-treated tectal membranes.

Using a similar bioassay in which stripes of temporal retina were confronted with alternative membranes from anterior and posterior tectum, the axons preferred to grow on the anterior membranes. As mentioned before, nasal axons did not distinguish between the membranes of either origin [359,360]. Staff and colleagues identified biochemically a 33-kDa protein called repulsive guidance molecule (RGM) associated with the posterior tectal membranes that accounted for the behaviour of the axons from the temporal retina by exerting a repellent activity [313]. RGM has now been cloned and is not homologous to ephrins, *Nogo* (IN-35), Slit or the netrins (Müller, personal communication). The protein blocks the growth of axons at low concentrations of 10 ng/ml, compared to 1 μg/ml of Ephrin-5. The first molecules that were unambiguously identified by their sequences and were proven to regulate retinal axon growth in vitro and in vivo were eph ligand (ELF-1), now referred to as Eph-A3 [47] and repulsive axon guidance signal (RAGS), now called Eph-A5 [78]. Similar to RGM, the Eph family of receptor tyrosine kinases are expressed in the retina and on the axonal growth cones [47,77,226,250,97]. Eph-A3 and Eph-A5 are expressed in gradients that increase along the nasal-to-temporal axis whereas Eph-A4 does not show a graded expression [51,47,48,78,226].

Ephrin-A3 expression is defined by the homeobox genes SOHO 1 and SH 6 along the naso-temporal axis [300]. Hornberger et al. [158] demonstrated Eph-A receptor function on ganglion cell axons by co-expressing Ephrin-A ligands and testing axonal growth in the stripe assay. In particular, temporal retinal axons lost their growth preference after overexpression of Ephrin-A5, whereas overexpression of Ephrin-A2 resulted in topographic targeting errors of temporal axons on the tectum. The gain-of-function and loss-of-function analyses carried out by these authors suggest a prominent role of axonally expressed Ephrin-A ligands [158,97]. The ligand–receptor interactions of ephrins are not restricted to one species. Brennan et al. [26] cloned two zebrafish cDNAs and expressed them in COS cells to show then that chicken RGC axons showed the same differential behaviour as when confronted with avian tectal membranes.

The data obtained in vitro with RGM and ephrins helped to propose a repellent mechanism of axonal guidance by expressing inhibitory molecules as predicted by the stripe experiments [358]. This mechanism is likely to act in a way that mediates collapse of the growth cone upon encountering non-favourable microenvironment. Indeed, avoidance responses that can alter the direction of axonal growth were induced by membrane-associated inhibitory molecules in vitro [59,358]. But withdrawal of retinal axons was also observed by a secreted tectal factor, that has not been identified further [166]. If similar mechanisms existed in vivo, that would mean that at decisive positions inhibitory molecules aid in the formation of distinct neural compartments by defining repulsive territories for subsets of arriving axons carrying incompatible positional information. Although no particular molecule has been characterised yet in vivo, the concepts derived from the bioassays in vitro point to a role of Ca^{2+}-dependent second messengers in growth cone movement [170,180,191].

4.2. Evidence for guidance mechanisms acting during normogenesis in vivo

In spite of the fact that vast numbers of data concerning guidance at molecular levels have been obtained in vitro, few of these observations could be transferred to explain the actual mechanisms of development in vivo. In the following, we will therefore dissect the knowledge obtained in vivo and try to compare it with hypotheses derived from culture experiments.

The matching between retinal development and the independent tectal cytodifferentiation attributes a particular role to the local expression and topochronolgical regulation of guidance cues: fine errors in the distribution of axonal guidance cues are thought to give rise to large perturbations of the topography. On the other hand, the autonomous development of retina and tectum raises the question of whether this hierarchy of genes is necessary or not. In the following, some experimental approaches will be discussed, which deal with alternations in the topography due to microsurgery (Fig. 5) and molecular manipulation in vivo.

Goldberg [119] rotated eyes at various developmental stages in the embryo in order to investigate the ensuing polarity of the optic fibres. He confirmed the model of autonomic eye development, since regional differentiation and fibre growth proceeded normally without axial realignment in the large grafts. In more recent experiments, Matsuno et al. [210] removed parts of the eye primordia at

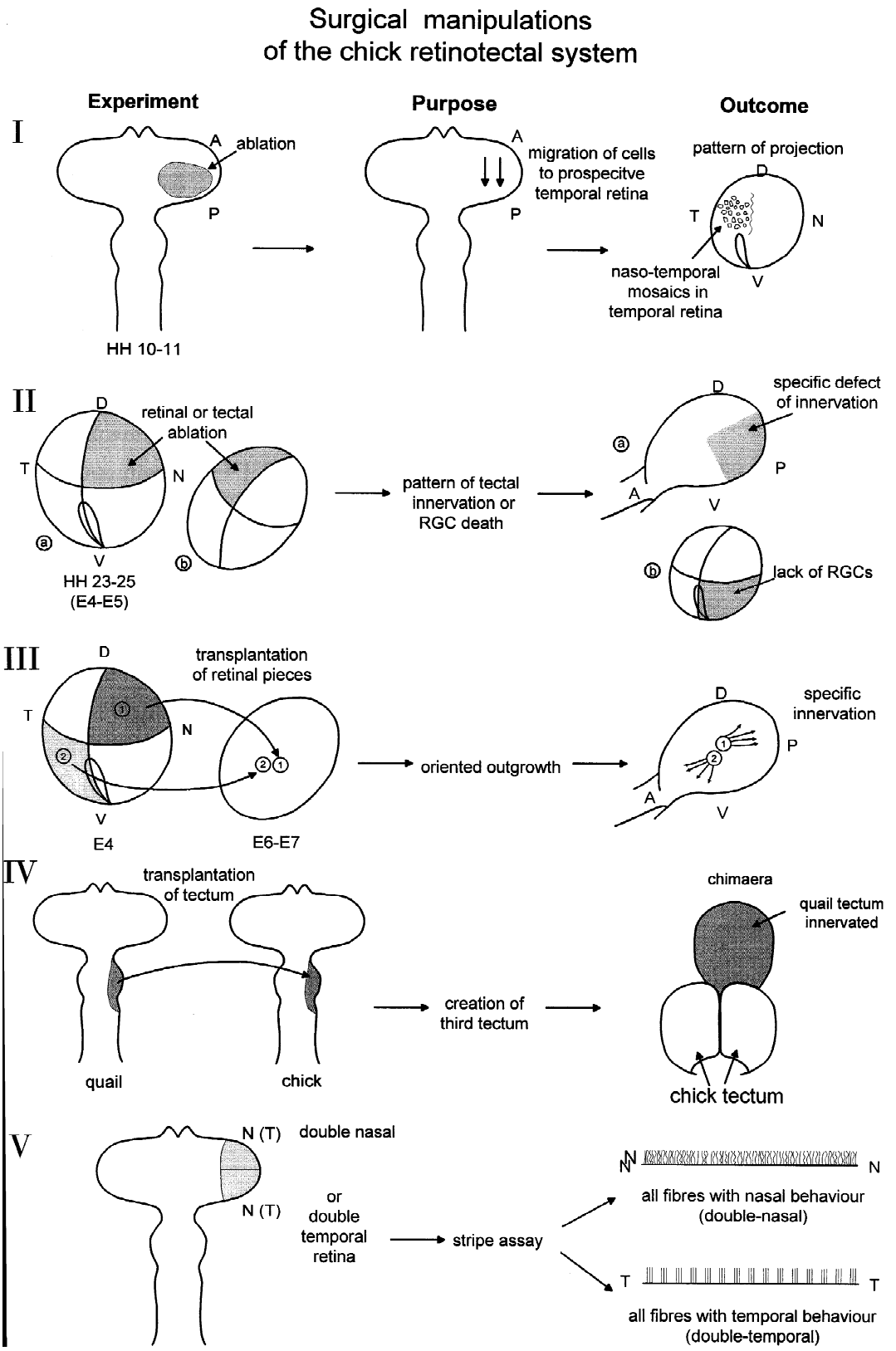

Fig. 5. Schematic drawings summarizing five examples of key experimental manipulations in the early chicken embryo in order to change the topographic representation on the tectum. The outcome of manipulations I and V are also documented in Fig. 6.

HH-13 to HH-14 and observed that the remainder of the optic vesicle formed a small eye. Neuroanatomical tracing revealed a detailed retinotectal map indicating that the positional specification was determined at the stages of surgery. These experiments are in line with the construction of bitemporal retinas in vivo which showed 'bitemporal' behaviour in the stripe assay [80] (Figs. 5V and 7D–F). Finally, ablation experiments [81,334] dated the time of positional specification at stage HH-10 to HH-11 (Figs. 5I and 7A), as later corroborated by investigations on the time of expression of transcription factors CBF-1 and CBF-2 [378] (Fig. 6).

The importance of the developing chiasm for the normal retinotectal topography was also first demonstrated in perturbation experiments. The relative accessibility of the chiasm allowed Fujisawa et al. [108] to surgically disorganise the chiasm at E3 and observe a changed retinotectal map as also observed by other deflection experiments [338,341]. An even more drastic manipulation was performed by Fawcett and Cowan [92] who divided the chiasm at E9 and observed a massive ipsilateral retinotectal pathway leading to formation of eye dominance stripes and patches in the double-innervated tectum. A non-invasive perturbation of the decussation pattern in the chiasm was achieved by intraocular injection of monensin, a specific inhibitor of membrane glycoprotein transport within the axons. This experiment suggested that the decussation is formed by neurite fasciculation mediated by membrane glycoproteins [236]. Retinal lesions also resulted in redistribution of nicotinic acetylcholine receptor subunits localised in the membrane [31].

Transplantation studies in chicken produced mostly negative or inconclusive results: after removal of the tectum, for example, fibres still grow to the position of their former target, pointing again to autonomous development of retina and midbrain (Fig. 5). de Long and Coulombre [66,67] were the first to transplant pieces of premature embryonic retina onto the tectum and studied establishment of a rough quadrantal topography. These experiments were repeated with fluorescence labelled retinal pieces injected into the superficial tectum to observe the trajectories of individual axons and the guidance to retinotopic areas of projection [340]. Alvarado-Mallart and Sotelo [3] transplanted tectal primordia from quail embryos to homotopic and heterotopic positions in chick embryos (Fig. 5). In some chimeras, a graft developed to a supernumerary third tectum at an ectopic position in the diencephalon. This quail-derived structure was innervated by retinal neurons from the chick; it competed with and even prevailed over the host tectum as a target for optic fibre terminals (Fig. 5). The authors assumed that this was due to chemoattraction by the ectopic xenograft. However, since the supernumerary tectum was integrated into the dorsolateral part of the thalamus in direct vicinity to the invading fibres, the deflected portion of fibres did not have to be attracted very far and long distance chemoattraction is not required to explain their results. Chemoattraction might cause the funnelling of axons towards the optic fissure within the retina, although circumferential intraretinal fibres seem exceptions in this regard [157]. Reviewing the data for neuronal chemotropism in various areas of the brain, Tessier-Lavigne and Placzek [333] concluded that the one molecule that has been most thoroughly investigated in this respect, nerve growth factor (NGF), does not appear to guide axons during development. They suggested that the following criteria should be met to demonstrate chemoaffinity as functional: secretion and diffusion of an attractant, thereby producing a gradient at the correct time and location in development, directed growth of axons as response to the gradient, and obligatory requirement of the attractant in vivo [333]. For the retinotectal system, long distance chemotropic guidance has not been demonstrated convincingly.

4.3. Molecular basis for retinotectal target specificity

4.3.1. Transcription factors (Figs. 6 and 9)

Based on ideas derived from the chemoaffinity hypothesis of Sperry, the spatial polarisation of retinal and tectal axes at predifferentiated stages (that is before E3) should coincide with the establishment of naso-temporal and dorsal-ventral gradients. Compelling evidence for an asymmetric expression of genes comes from the analysis of CBF-1 and CBF-2 genes along the prospective naso-temporal axis [378] and for T-box-transcription factor gene 5 (Tbx-5), Vax and Pax-2 along the prospective dorso-ventral axis [188]. The differential expression of CBF-1 in the prospective nasal and of CBF-2 in the temporal neuroepithelium was in line with the ablation and transplantation experiments aimed at manipulating the eye primordial to investigate the topographical outcome [80,81,334] (Fig. 6A). The instructive role of CBF-1 and CBF-2 was demonstrated in over-expression experiments in young chicken embryos and the subsequent examination of the retinotectal map that was altered [378]. Although not decisively associated with the protein patterns, it is likely that the asymmetric activation of the transcriptional factors CBF-1 and -2 results in asymmetric down-stream cascades with asymmetric distribution of guidance molecules and cell identity as the final outcome (Fig. 6). These experiments were also in line with the remarkable differences in the protein patterns when primordial halves were analysed with 2D-SDS-electrophoresis and autoradiography revealing an asymmetric expression of proteins [334]. At the mRNA level Godbout [117] identified transient transcripts with asymmetric distribution in the undifferentiated retina. One of these was identified as cytosolic aldehyde dehydrogenase [118,223]. These data were in line with similar discoveries in the mouse, where the search for asymmetrically distributed molecules led to the discovery of a complicated pattern of retinoic acid synthesising and degrading enzymes [212,214] (see below). By activating

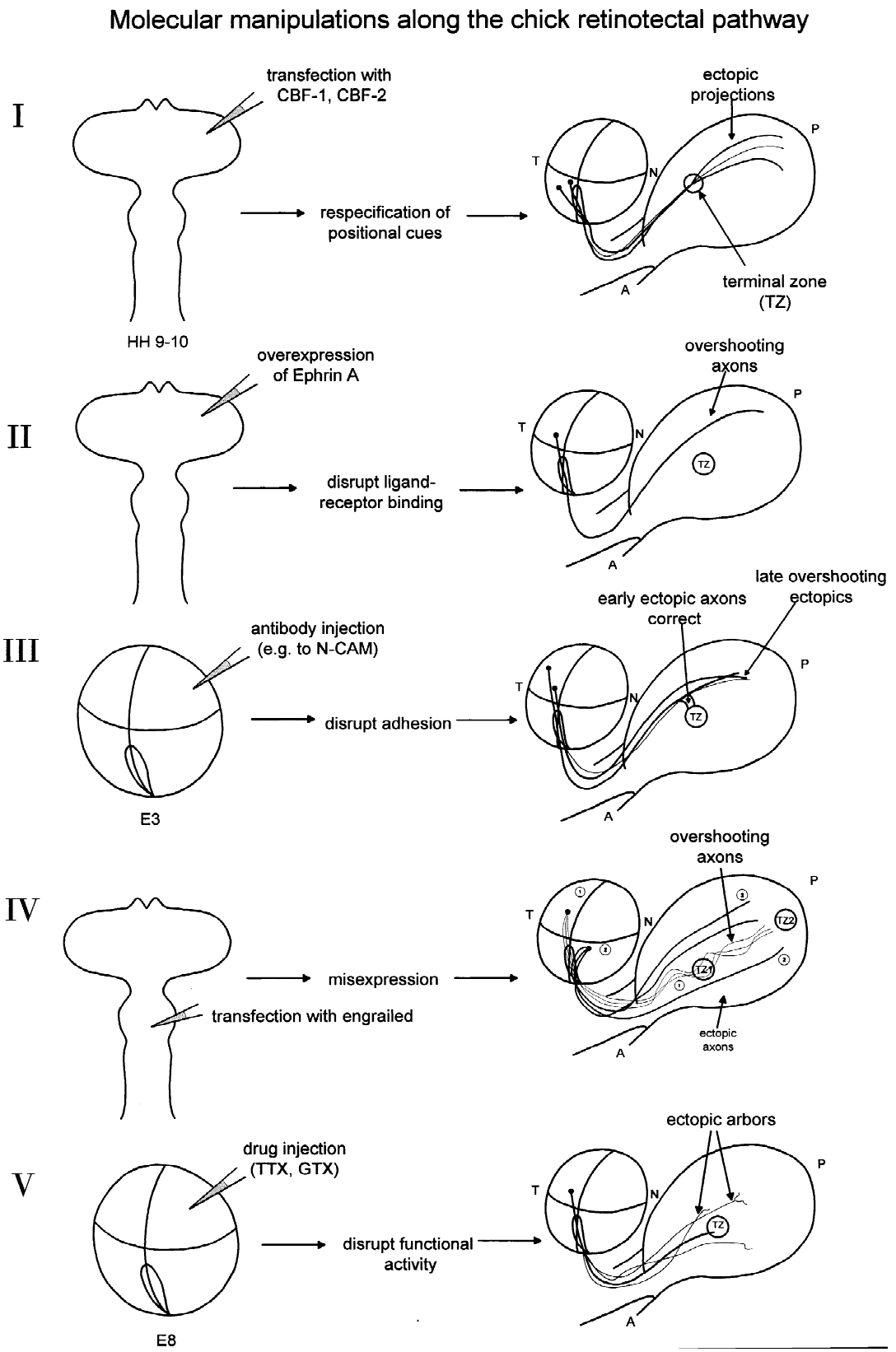

Fig. 6. Molecular manipulations along the retinotectal pathway include transfections with CBF-1 and CBF-2, transfections with ephrins, and injections of antibodies to cell adhesion molecules, or to repellent molecules. The outcome of each experiment is schematically shown in the accompanying graphs.

nuclear receptors, which then bind to enhancer elements on the DNA, retinoids may even act upstream of the transcription factors that were actually shown to determine polarity along the dorsal-ventral eye axis.

Candidates are Pax-2 and Vax in the ventral [300] and Tbx-5 in the dorsal retina [188]. Misexpression of the Tbx-5 gene with in ovo electroporation caused dorsal spatial identity of the whole retina, as indicated by repression of ventral markers including transcription factors Pax-2, Vax and putative guidance molecules Ephrin-B1 and Ephrin-B2 (Fig. 6). Anterograde and retrograde neuroanatomical tracing demonstrated that in several of these embryos axonal trajectories from the ventral retina were deflected from their paths to tectal destinations corresponding to dorsal fibres [188].

4.3.2. Molecular gradients with uncertain functions (Figs. 6 and 9)

The dominating strategy of the 1980s was to raise antibodies against cell surface molecules that occurred in gradients across the surfaces of embryonic retina and optic tectum. One such group of asymmetrically distributed molecules were the toponymic (TOP) proteins [343–346]. In the embryonic retina the toponymic dorsal-ventral (TOP_{DV}) antibody recognised a dorsoventrally graded molecule of ~47 kDa with a more than 30-fold higher concentration in the dorsal retina [346]. The distribution of TOP_{DV} is inverted within the tectum again rising in a graded fashion, here ten-fold from dorsal to ventral. Expression of TOP is detectable during the major period of fibre arrival and innervation of the tectum, but drops continuously between E10 and E18 [345,346]. A second antigen of 40 kDa was distributed along the nasal-temporal axis (toponymic anterior-posterior, TOP_{AP}) with highest concentration within the temporal retina and the anterior tectum [293,343–346]. Although no function could be attributed to the TOP-antigens yet, the topography-related asymmetric distribution both within the retina and the tectum indicates a direct or indirect involvement in coorienting the retinotectal map. The fact that even 20 years after discovering the TOP-antigens their function remains obscure casts doubt, however, on their direct or crucial role in controlling of the precise retinotectal projection. Judged by the knowledge accumulated within the last years, a number of secondary gradients with yet unknown functions may be inferred in the developing pathway.

The difficulties in defining functions on the basis of the antigen distributions also became apparent in the case of another molecule, targeted with monoclonal antibodies. McLoon [220] described an antibody ‘TRAP’ that recognised an antigen of 135 kDa, distributed with increasing concentration within the temporal retina. In contrast to TOPs, ‘TRAP’ showed not a graded, but a ‘step-like’-transition in the retinal midline. The antibody TRAP bound to retinal fibres in culture and was expressed in growth cones. However, no function could be attributed to TRAP yet, and no clear role could be established.

Two other monoclonal antibodies, ‘Julia’ and ‘Dolce’, bind preferentially to the developing dorsal retina of chicks, mice, rats, and *Xenopus*. They recognise a 68- and a 44-kDa protein. The 68-kDa protein and its mRNA are found throughout the whole retina, but the molecule seems to occur in a different configuration in the dorsal retina [272].

In an attempt to find axial differences along the dorsal-ventral axis of the embryonic mouse retina McCaffery, Dräger and colleagues hit upon an abundant protein in the dorsal retina. This turned out to be an aldehyde dehydrogenase, an unlikely candidate as a guidance molecule [212,213]. However, this enzyme synthesises retinoic acid, which is a potent transcriptional activator of many regulatory genes, including transcription factors and signalling molecules of various families. In the chick eye, three retinoic acid producing enzymes were identified, two of which are contained in the neural retina and distributed asymmetrically along the dorsal-ventral axis. Chromatographic analysis showed a higher concentration of retinoic acid in the ventral retina [223]. Indirect measurements of retinoic acid activity obtained with mice [214] and chick retina (J.M., unpublished observations) indicate, however, that retinoic acid concentration is indeed highest in the ventral half of the embryonic retina, but that it declines toward a horizontal zone in the middle and increases again towards the dorsal pole. This distribution is not easily reconciled with the demands of the chemoaffinity hypothesis. Moreover, attempts to show retinoic acid gradients at E2, when the determination of retinal polarity takes place have not been successful.

Further experiments in vitro specified the repulsive character of posterior tectal membranes to fibres from the temporal retina: the effect, tested with growth cone collapse and the stripe assay, was destroyed by treatment with proteases, heat, and phospholipase C, suggesting that the molecule is a protein, anchored with phosphatidylinositol to the membrane [59,358–360]. Antibodies were raised against purified tectal membrane extracts, and biochemical analysis led to the discovery of a 33-kDa glycoprotein, called RGM (repulsive guidance molecule) that is expressed in higher concentration on the posterior surface of the tectum than anteriorly [313] (Fig. 9). The gene of this molecule has been sequenced and its expression found to be developmentally regulated. RGM blocks growth cone movement at much lower concentrations than Ephrin-5 (Müller, personal communication).

4.3.3. The ephrin system (Figs. 6 and 9)

The group of putative guidance molecules that is most intensely studied at present are the ligands for Eph-related tyrosine kinase receptors, which have first been characterised outside of the retinotectal pathway [12,14]. The Eph family of receptor tyrosine kinases are also found in

gradients across the early retina of the chick and on the growth cones of ganglion cells [47,48,226]. Two subfamilies are recognised, Eph-A (A1–A8) and Eph-B (B1–B6), based on their affinity to two corresponding subsets of ligands. Ephrins-A (A1–A5) are anchored by a GPI linkage to the plasma membrane, and Ephrins-B (B1–B3) by a transmembrane domain. With few exceptions Ephrins-A activate all Eph-A receptors, Ephrins-B all Eph-B receptors [88].

In the chick optic tectum Ephrin-A2 and Ephrin-A5 are distributed in gradients (Fig. 9), increasing from anterior to the posterior pole, with Ephrin-A5 being restricted to the caudal half of the tectum. The preferred receptor for these ligands, Eph-A3 is present in a retinal gradient, increasing from nasal to temporal [47,48,78].

Along this axis, it seems that the tectal distribution of Ephrin-A2 and possibly Ephrin-A5 is a repellent guidance system that stops growing retinal axons at different anterior-posterior positions according to their expression of Eph-A3 on the growth cones. This concept was corroborated with in vitro analyses using the stripe assay and with experimental Ephrin-A2 misexpression in the developing tectum [47]. The ligands co-localised with the receptors on RGC. Overexpression of the Ephrin-A ligands on temporal retinal axons abolished their repulsive sensitivity in the stripe assay and resulted in topographical errors of temporal axons in vivo. An inverted sensitivity was observed with PI-PLC treatment of nasal axons, stripping them of the Ephrin-A ligands, thereby rendering them sensitive in the stripe assay [158] (Fig. 7B,C). A similar effect was also observed by von Boxberg et al. [354], who used a different protocol of preparing the tectal membranes. In the chick retina the tyrosine kinase receptors Eph-A4 and Eph-A5 are uniformly expressed.

In the murine retinocollicular pathway, Eph-A4, Eph-A5 and Eph-A6 are also expressed in the retina, with Eph-A4 being distributed uniformly and Eph-A5 and -A6 increasing in nasal-temporal gradients [34,95]. Ephrin-A2 and Ephrin-A5 together form a smooth gradient of axon-repellent activity across the superior colliculus of the mouse [34,104]. In the absence of both of these ligands the retino-collicular topography was severely disturbed [94]. In a recent experiment, the Eph-A3 receptor, which does not occur normally in the mouse ganglion cell layer, was upregulated in a subset of mouse retinal ganglion cells. This resulted in two intermingled RGC populations that expressed distinct Eph-A receptor gradients [34]. As expected from the model, the authors found that in addition to neurons with close to normal projections, one population of RGC projected to a more rostral position on the tectum. Thus, anterograde tracing experiments in homozygous knock-in mice yielded two parallel retinocollicular mapping functions, and in situ hybridisation confirmed that RGC with an anterior shift in their termination zones expressed Eph-A3 ectopically. In addition, since these cells displaced wild type RGC from normal rostral termination sites, it was not the absolute levels of Eph receptor expression but relative levels compared to that of neighbouring RGC terminals that appeared to determine the topographic pattern of the projection. This is indicative of a competitive mechanism between RGC axons [34]. Specific ligands for Eph-tyrosine kinase receptors, like AL-1/RAGS/Ephrin-A5, were found to be involved in axon bundle formation [371]. Peles et al. [260] identified that the carbonic anhydrase domain of the receptor tyrosine kinase beta is a functional ligand for the axonal recognition molecule contactin.

Along the dorsal-ventral axis the subset of Ephrin-B ligands and their receptors might be relevant guidance cues, because some of these molecules are spatially segregated along this axis [250]. Braisted et al. [24] found graded and lamina-specific distribution of the ligands of Eph-B receptor kinases in the developing retinotectal system, and the kinases were proposed to be directly involved in axonal guidance [25,250]. More recently, Birgbauer et al. [16] found a differential kinase-independent response of dorsal and ventral retinal axons. Ephrins-B1 and -B2 occur in the dorsal retina and are upregulated by Tbx-5. In contrast, expression of the receptors Eph-B2 and Eph-B3 in the ventral retina are repressed by this transcription factor [188].

The complexity of the developing retinotectal system in vivo can probably not be assessed by describing one single class of molecules. Laborious experiments of the last few years suggest indeed that hierarchically organised cascades account for the complexity of making a projection, because surface proteins are the ultimately functional members of decisive cellular events. Transcriptional regulators form the initial members of such chains. The transcription factor *engrailed*, originally described in *Drosophila*, is represented with homologous genes *en*-1 and *en*-2 in vertebrates [110]. Rotation and grafting experiments and creation of chick-quail chimeras revealed a topographically relevant, and boundary-determining expression of the *engrailed* gene in the tectum [2,208]. The grafting experiments of Itasaki and Nakamura [168] and Itasaki et al. [169] correlated the establishment of rostral-caudal midbrain polarity with expression of *engrailed* in a graded and topographic order. In accordance with these experiments *engrailed* is expressed highest in the caudal tectal primordium before HH-25, that is before fibres innervate the tectum. In accordance with the studies on repellent molecules, temporal retinal axons avoid tectal regions with high expression of *engrailed* [103,168]. Hence, there is a correlation between avoidance of the posterior tectum, expression of *engrailed*, and overexpression of Ephrin-A2 and Ephrin-A5 in this region [158,168,169,199] (see also Fig. 9 for a summary).

The crucial role of *engrailed* seems, however, to be the definition of regional specializations by regulating the expression of diencephalic Pax-6 that preceded the induction of mesencephalon-related genes like Pax-2, Pax-5,

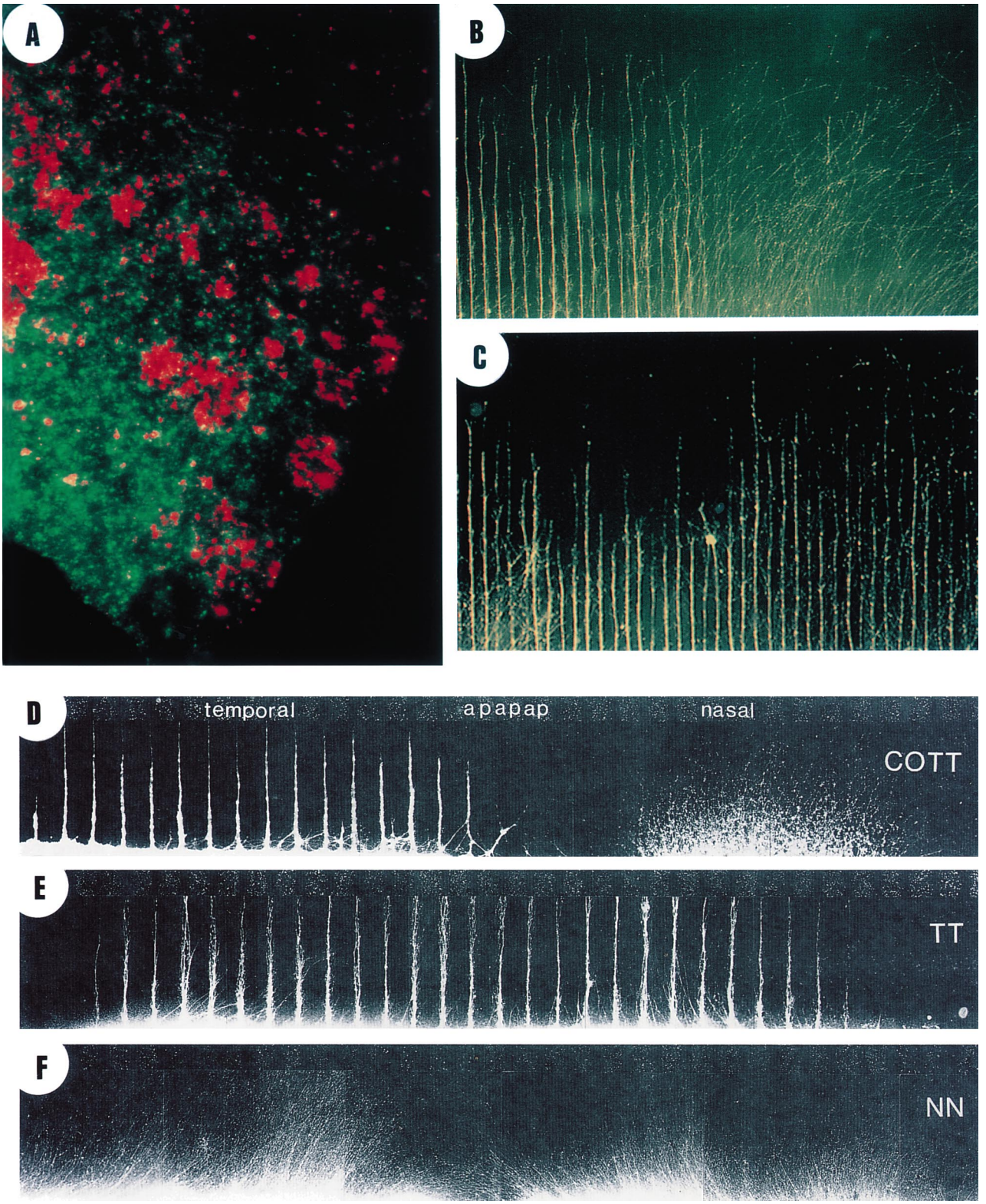

Fig. 7. (A) Retrogradely labelled mosaic retina showing mixed RGC with nasal (red) and temporal (green) projection properties after the manipulation shown in Fig. 4I. (B) In the classical stripe assay temporal retinal axons (left) show a preference for membranes deriving from the anterior tectum, whereas nasal axons (right) do not discriminate between the alternating stripes of membranes from anterior and posterior tectum. The retinas were transfected with Ephrin-A2. (C) Treatment with PI-PLC resulted in sensitivity of nasal axons to Ephrin-A2 and in bundled growth in the stripe assay, indicating that the Ephrin-A2-overexpression operates through PI-PLC anchors. (D,E,F) Creation of bitemporal (TT) or binasal (NN) primordia resulted in retinas with either completely temporal (E) or completely nasal (F) behaviour in the stripe assay. Compared to this, the normal retina (D) shows the typical naso-temporal differences. The images correspond to the manipulation shown in Fig. 5V.

fibroblast growth factor 8 (FGF-8), Wnt-1 and Ephrin-A2 [5]. The positional specificity of the tectum is also controlled by Grg-4 that is early expressed (HH-10) in the mesencephalon and causes repression of En-2, Pax-5, Fgf-8 and Ephrin-A2 [324] (Fig. 9). The cross-regulation of a number of genes within the diencephalon and mesencephalon seems therefore crucial in defining the rostrocaudal polarity, and resulting in guidance of axons along this axis [221]. It is obvious that future studies will need to consider this hierarchical interplay of genes and systematically analyse mutants and overexpressing embryos in order to define the way it really works. The cellular elements expressing the aforementioned genes still remain to be analysed. In a first attempt to localise the positional cues to a specific cell type, Gray and Sanes [124] ascribed to the tectal radial glia an involvement in axonal guidance by using grafts of clonal retinal cells which resulted in specific migratory paths and phenotypic choices dependent on the clonal origin.

5. The attempt to match theoretical predictions with experimental data

5.1. Mechanical models of topography

One of the basic features of visual field representation is the precise cellular topographic projection of neighbouring ganglion cell axons on the tectum. Ganglion cells residing in close proximity will send axons to neighbouring tectal neurons thereby keeping the coordinates both in relation to each other, and in relation to the entire visual field represented by the total number of RGC. However, neither the area of origin (retina) nor the target (tectum) provides a simple, two-dimensional surface. Both organs have different curvatures, metric coordinates and sizes. It is therefore not simple to devise a Cartesian model that unifies all properties to sufficiently explain how the projection is formed (Fig. 8).

A variety of models have been proposed [121]. One concept that is independent of the requirements of the chemoaffinity hypothesis postulates that axons from RGC born early in development arrive first, form connections first and exclude or inhibit subsequently arriving fibres from setting down at this position [273,274]. This order-in-time can be transformed to order-in-space with more peripheral fibres left to occupy more peripheral tectal positions which are still empty. This model would be applicable to one spatial dimension (anterior-posterior axis). Organisation of the second axis (dorsal-ventral) would result from the pre-existing anatomical order of axons within the optic nerve, tract and fibre layer on the tectum (Fig. 8). The assumption required a rather linear wave of birth of ganglion cells and growth of their axons. Central neurons and axons precede the peripheral cells, which have to be added in a circumferential fashion. This postulate was already difficult to reconcile with experimental evidence about the sequence of RGC birth and RGC differentiation. The central area is at least 2 mm away from the site where the first RGC were generated, and it is not before stage HH-28 that the prospective area centralis is populated by RGC [280]. In addition, the retina increases in size asymmetrically, and new cells are not only added in the periphery, but also within areas of pre-existing cells.

The order-in-time model makes further predictions. First, retinal ganglion cells are generated in a central to peripheral succession, and in that sequence their axons arrive at the optic fissure with locations and angles that also depend on the site of their cell somata. As a result, fibres are placed in the optic nerve in a chronotopic order that corresponds to the spatial order of the ganglion cells [119,275,336]. Second, the regional maturation of the tectum proceeds in a sequence that exactly matches the retinal ganglion cell differentiation. Rager describes that the rostral-central region, which has to receive central retinal axons, is the first tectal area to develop appropriate receptive structures, concluding that the earliest fibres grow until they encounter mature dendrites on the tectal surface. Later, axons are added successively according to their position in the optic tract [275,277]. It is of critical importance for this model that the retinal neighbourhood relations are preserved and appropriately transformed in the optic pathway. The transformation has to provide for that specific mirror imaging of the ventral retina to dorsal tectum, temporal retina to anterior tectum etc. Rager stresses that the precision required in the retinotectal connection only needs to be in the order of fibre fascicles, not of single cells [275].

How are these predictions supported experimentally? Ehrlich and Mark [86] made laser lesions in the retina and examined the pattern of fibre degeneration. They found that the central-peripheral RGC succession of the retina is represented along the rostral-caudal axis in the optic tract. On the other hand, temporal and nasal fibres seemed to be mixed. In contrast, Rager himself and his co-workers never observed a mixing of nasal and temporal fibres [279]. An additional, contradictory fact, namely that many fibres enter the tectum at an incorrect position along the dorsal-ventral axis, has been corroborated by others [235]. An investigation by Rager's group of the axonal pathways along their course established that transformations of the retinotectal topography follow particular constraints: in the optic nerve head, fibres are mirrored across an axis extending from dorso-temporal to ventro-nasal retina. Afterwards, only minor changes occur, namely clockwise rotation behind the chiasm, and flattening of the whole tract. This leads to an organisation of fibres when they arrive at the tectum, which fulfils the requirements for Rager's model [279]. Additional support for the maintenance of order may come from preferential mutual adhesion by neighbouring fibres [7]. The rough order of the projection will be refined later [276]. In view of the advent

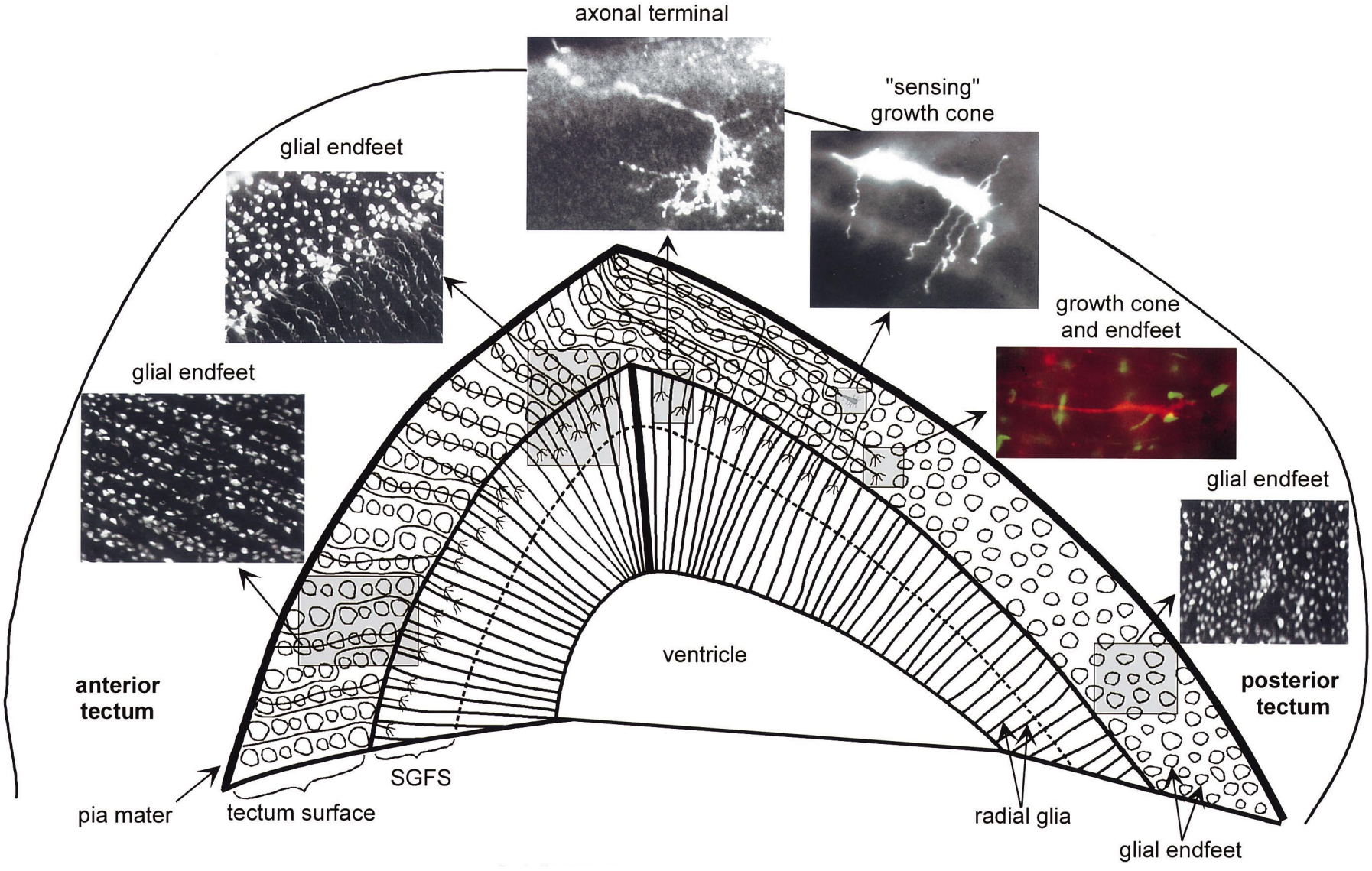

Fig. 8. (A) Three-dimensional reconstruction of the superficial optic tectum at ~E9 to E10 with the radial glial fibres extending from the ventricle to the surface. Retinal growth cones intermingle with the glial endfeet to then invade the SGFS and terminate within its sublayers. Attached photomicrographs show the microenvironment which interferes with ingrowing retinal axons. In particular, the organization of glial endfeet is shown in the tectal surface, as their distribution- and innervation-dependent arrangement may carry the guidance cues. Typical growth cones with perpendicularly oriented filopodia and axonal terminals are shown to illustrate the structural correlates of pathfinding.

of molecular tools at a later time, the mechanical model of Rager lost its importance. It becomes increasingly apparent that molecular guidance is required and phenomenology is secondary to that guidance. So far, the time-in-order predictions are set by basic mechanisms of guidance and do seem instructive for the precise projection.

Because the retinotectal system is considered mature at the time of hatching (P1) it is hard to assume that any of the newly added cells are RGC. Waid and McLoon [355] analysed the production of new RGC in retinal cultures of various ages and found that older ganglion cells secrete a factor which inhibits production of new ganglion cells through a pathway which is different from that mediated by *Notch*. In their earlier investigation based on retina wholemounts Straznicky and Chehade [320] had identified preferential cell death in the periphery and differential retinal expansion as the most important mechanisms in development of the area centralis. They also showed that the regional and temporal sequence of nuclear pyknosis followed the pattern of general retinal differentiation (central to peripheral, temporal to nasal). The death of retinal neurons including ganglion cells depends on NGF acting via the P75 receptor at early stages [100]. While NGF- or NT-3 addition had no effects on preventing ganglion cell death, BDNF appeared an effective neurotrophin resulting in ~70% increase in the number of RGC at E6 to E9 retinas [99]. The regulatory role of BDNF was also associated with terminal axonal branching of transiently projecting retinal-retinal ganglion cells [335]. Developmental neuronal death is, however, not a universal event among the various cell types in the chick retina. As shown by Cook et al. [56] cell death affects mainly the GCL and inner nuclear layer (INL), whereas no pyknotic or terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL)-positive cells were observed within the ONL.

Modifications of the order-in-time/order-in-space model are compatible with additional fibre/fibre interactions along the pathway and imposition of markers on the tectum that can be selectively read by properly arriving axons. In this way, the tectum acquires markers which depend on innervation by retinal axons. Such a self-regulating model was proposed to include competition [268,369], and extended [365] to include synaptic plasticity as basic element of local regulation in the tectum.

5.2. Chemoattractive guidance by gradients encoding complementary positional cues

To explain such 'homing' behaviour of nerve fibres, Sperry postulated a growth mechanism that would selectively establish synaptic associations independent of function and be regulated by specific cytochemical affinities. While the early articulation of the theory required literally millions of chemically distinguished cell types alone for the retina, that postulate of biochemical labels for every individual neuron has been abandoned in view of the limited amount of available genetic information. Sperry reformulated the theory proposing ''an orderly cytochemical mapping in terms of two or more gradients of embryonic differentiation that spread across and through each other with their axes roughly perpendicular. These separate gradients successively superimposed on the retinal and tectal fields and surroundings would stamp each cell with its appropriate latitude and longitude expressed in a kind of chemical code with matching values between the retinal and tectal maps'' [312].

In the light of accumulating empirical data about retinotectal systems in all classes of vertebrates, Sperry's theory has been widely accepted and elaborated [121,317]. Fraser and Perkel [102] constructed a model that is centred around the chemoaffinity principle, but integrates several mechanisms: while selecting their targets, retinal growth cones are guided by mathematically defined multiple constraints. These conditions are: (i) position-independent affinity for the tectum, (ii) competition for synaptic space, and (iii) position-dependent affinities of the optic fibres to appropriate tectal targets. The latter position-dependent adhesion is assumed to be coded in two gradients, one along the dorso-ventral axis and one in anterior-posterior direction. Computer simulations allow generation of normal retinotopy and experimental results obtained in grafting and ablation experiments [101,102]. The central issue in chemoaffinity remains, how the position specificity is encoded and by which molecular mechanisms axonal growth cones orient themselves in the target area. The programme developed by Gierer [113,114] assumes that axons respond to spatially graded concentrations of guiding substances. Gradients are required for both dimensions of the tectal surface, and along each axis two counter-graded effects are necessary to create target positions inside the fields for each fibre terminal. These antagonistic effects depend quantitatively on molecular components of the axonal growth cones like receptor concentrations or modulated receptor affinities. To designate different specific locations along the gradients for individual axons by this system, the biochemical outfit of different growth cones themselves must be graded according to their retinal origin [102,114]. The physical mechanism for axonal guidance may be a force of maximal adhesion to the substrate [197] or, as it is most often assumed, chemoattractive and repulsive influences [34,114,78]. In this case, the problem arises, how the relatively small growth cones can detect slight spatial gradients by extending some filopodial protrusions at costs to others (Fig. 4, and growth cone insert in Fig. 8).

One possibility is a temporal detection of the gradient, as used in bacterial chemotaxis. As another solution, an adaptation of growth cone sensitivity to repulsive influences has been suggested [18]. In analogy to the cAMP-chemotropism of cellular slime moulds, Gierer conceived a mechanism that secondarily enhances a slight external

gradient within the growth cone (similar to the signal transduction shown in Fig. 4). This amplification might result from receptor-mediated short range autocatalytic reactions in combination with lateral inhibition or depletion. Thus, a restricted focus of activity would be created at the growth cone leading to oriented fibre growth or even axonal branching (Fig. 4). A computer model, based on these principles, simulated empirical growth cone pathways [114]. It assumed just one or two guiding substances with a graded distribution both within the retina and the tectum. The conditions of this model are simple and include the slope of the guiding substance, conventional enzyme-receptor kinetics, potential but not necessary activity of adhesion and polarised distribution within the growth cone to activate its polarity and direction of growth [113,114]. An extension of this gradient-model includes repulsion at inappropriate areas of termination as a mutual component of making the topography [114]. Irrespective of whether the growth cone reads inhibiting or attractive cues (as imposed in Fig. 4), it can integrate guidance information over long distances, perhaps by combining memory and/or adaptation mechanisms. Such integration was experimentally assessed with experiments confronting ganglion cells axons with precisely formed striped linear gradients of repellent tectal membranes and of two repellent molecules, Ephrin-A2 and -A5 [289]. The molecular mechanisms of intracellular signalling that translates external guidance cues into directional movement of the growth cones are a topic of current research in various areas of the CNS [83,204,290,331,332] (Fig. 4).

While theoretical considerations and several in vitro assays all point to guidance of fibres by interaction with local, membrane bound cues according to the chemoaffinity hypothesis, the experimental evidence is much less clear. Supporting data are largely indirect. Thanos and Bonhoeffer [336,339] employed anterograde labelling techniques to demonstrate axonal growth and branching patterns on the tectal surface: a majority of axons course along direct routes. Some apparently ectopic axons, which occur in normal development and after experimental disturbance, seemed to have corrected their path along the dorso-ventral axis in a manner that is consistent with Gierer's model. This is especially found in early arriving fibres [341], which, additionally, display more complex growth cones than the later arriving ones. It is therefore likely that the first axons orient themselves in a field of positional cues, while following fibres fasciculate with pre-existing axons that belong to neighbouring ganglion cells. The close arrangement of ingrowing axons and radial glia led to the suggestion that positional cues are expressed on glial endfeet [125,352]. Still, these observations did not distinguish whether turning growth cones are attracted or repulsed.

The second line of evidence that lends support to the chemoaffinity but not to gradients derives from in vitro studies on retinotectal affinity. Measuring cell–cell adhesion between dissociated retinal cells and tectal tissue in vitro, Barbera [10] discovered that cells from dorsal retina adhered preferentially to ventral tectal halves and vice versa. In addition, the effect seemed to be developmentally matched with the time course of the process in vivo: ventral retinal cells, for instance, expressed the preference for the dorsal tectum only when dissociated after E6 [10]. A variety of other in vitro models on regional specificity have since then been developed. They examined retinal cell–cell adhesion [123], tectal membrane attachment to retinal neurites [138], retinal neurite growth on monolayers of tectal cells [21], or membranes [360], stimulation of neuritic growth by tectum extracts [42], and growth cone collapse induction by tectal membrane preparations [59]. These systems often could detect some preference of retinal neurites towards corresponding parts of the tectum. For example, the growth of (E6) nasal fibres is best stimulated by extracts from (E18) anterior tectal halves [42].

All previous in vitro experiments, however, failed to bring forth conclusive evidence for a gradient model, because (i) they were far too unspecific to detect any preference that could account for target specificity in the chemoaffinity model [10,42,59,123], or (ii) they failed to reveal any *graded* characteristics along the tectal axes instead of a sharp transition [9,138,358–360], and (iii) often differences were detected along one axis only but not along the other [21,59,138,360]. The most convincing results in vitro were then obtained with the assay developed by Bonhoeffer and co-workers [359,360]. Growing retinal neurites are offered membrane carpets from different tectal origin on alternating stripes. It turned out that temporal retinal axons avoid posterior tectal membranes when given a choice, but are still able to grow almost equally fast on tectal membranes of whatever origin [358,360]. This preference seems to be caused by a repellent substance rather than differential attraction, because prior treatment of posterior tectal membranes with heat or proteases converted them into an equally good growth substrate as anterior membranes [359]. Axons from nasal, dorsal, and ventral retina showed no preference.

As discussed above in Sections 4.2 and 4.3, the most convincing results for physiological functions of gradients of guidance molecules were obtained in vivo. Specific overexpression of Eph receptors resulted in altered projections largely consistent with postulations of the chemoaffinity hypothesis [34].

5.3. Lessons from retinotectal systems in other species

5.3.1. Drosophila

Since the molecular mechanisms which control positional identity in the organism's bodyplan are best studied in *Drosophila*, the concepts derived from *Drosophila* neurogenetics were used to gain insights into general mechanisms of vertebrate development as well. Homologies have

been found to extend even to the presence of individual genes including intercellular signalling pathways and a large number of transcription factor genes which were analysed in vertebrates [217]. Some of such homologues are expressed in the developing CNS [270]. As a first example, *engrailed*-1 (*en*-1) and *en*-2 showed a graded expression in the avian tectum [110]. As observed later, engrailed-expression controls the positional specification and determines the ephrin-dependent navigation of axons [158]. It is a decisive recognition that the conserved specification of the antero-posterior polarity of the body has crucial effects on axonal pathfinding. Ectopic expression of *en*-2 resulted in development of tectal cells which acquire 'caudal' positional specificity, and these cells may express the ligands for Eph type receptor tyrosine kinases [199,304]. The predictable readjustments of the retinotectal map after expression of *engrailed* points to its high level of hierarchy within the molecular cascade. However, *engrailed* is set up by FGF-8 and WNT-1, two further *Drosophila*-derived genes [285]. As a second example, the winged-helix (WH) domain is a DNA-binding motif in the transcription factor family and contains *Drosophila forkhead* and vertebrate HNF-3 [178]. This shows a strong homology to rat-brain-factor-1 (BF-1) and to chick CBF-1. The temporal-retina-specific CBF-2 is the chick homologue of rat brain-factor-2.

A third example is the conserved appearance of RTPs, a family of *Drosophila* receptor tyrosine phosphatases required for guidance of motor axons [318]. The expression of the RTP ligand contactin/F2/F3 on retinal axons, of RTP_{β} on glial cells, and of CRYPα on axons and growth cones [195,318] is consistent with the idea that such kinases play a role in axonal guidance in the chick. Also *Six-3* and *Six-6(OPTX-2)* are murine homeobox-containing genes which belong to a separate group of genes that are closely related to *Drosophila optix* [172]. *Six-6* acts on a parallel and/or independent pathway with *Pax-6* in the genetic cascade controlling development of the eye. *Six-3* is the homolog to *Drosophila sine oculis* and associated with a number of developmental abnormalities in the mammalian eye [200]. It is to be expected that numerous further genes first analysed in *Drosophila* will enter the research on CNS development in vertebrates.

5.3.2. Zebrafish

Phylogenetically, the retinotectal projection is similarly organised from fishes to birds and 'lower mammals' like rats and mice [160]. It is therefore not surprising that very similar mechanisms operate during development. The zebrafish *Danio rerio* has only recently entered the search of molecules involved in setting the retinotectal map. The normal development of this projection is known [39,322,347]. Unlike the chicken and all other vertebrates, the zebrafish offers the possibility of genetic approaches like mutagenesis as this has been studied in *Drosophila*. Consequently, it offers the opportunity to characterise new, vertebrate-specific genes which may control the genesis of the retinotectal map. The screening of mutants after point mutations revealed indeed a disrupting of the retinotectal order and the topographic projection [347]. Until 1997 over 30 genes were found to affect either axonal pathfinding or the topographic connection between the eye and tectum [179]. The laboratory of Harris and colleagues discovered 400 zebrafish mutants which displayed histological, behavioural and other deficits. This approach will certainly help to genetically dissect the vertebrate visual system [244] with direct consequences for the development of chick visual projection. But the high numbers of genes resulting in defective phenotypes point to the complexity of the question of how the map is made.

The identification of several retinoic acid producing aldehyde dehydrogenases with spatial restrictions along the dorsal-ventral axis of the eye suggested that a retinoic acid gradient may determine retinal polarity along this axis [212,213]. While for the chick retina only indirect evidence exists, namely an asymmetric distribution of retinoic acid [223], experimental support for this concept has been obtained in zebrafish. Results from retinoic acid treatment or inhibition of aldehyde dehydrogenases in zebrafish larvae in vivo were interpreted as evidence for a ventralising influence of this molecule on retinal polarity [212,213]. Although retinoic acid treatment disturbed fibre growth in *Xenopus* to this day there are no reports that would demonstrate a decisive effect on development of the retinotectal topography in any vertebrate.

5.3.3. Goldfish and amphibia

It is noteworthy that the chemoaffinity hypothesis was formulated after experimentation in *Xenopus* [312]. Most of the studies of the well-known and relatively similar visual systems of fishes and amphibia have been performed in the regenerating system after injury to their optic nerves [105,112,321,323,348], although their normal development has also been studied in detail (tadpoles [101,107,330], goldfish [15]). More recently, several lines of evidence point to an activity dependent development and sharpening of these projections [174,255,299]. However, the molecular analysis of these systems is less advanced than that of the chick, although molecules like Slit are also expressed in the *Xenopus* embryo to be then secreted into the extracellular space [49]. The fruitful lessons remain therefore in the consideration of activity-dependent co-development, of regeneration that does not occur spontaneously in the chick, and in setting the theoretical models. The experimental set-ups are similar to these in the chick and involve perturbations with antibodies to cell adhesion molecules [319] or blockade of synaptic transmission [253]. A recent electrophysiological study demonstrated activity-dependent cooperation and competition of convergent retinotectal synapses in *Xenopus*. To serve as a mechanism for the refinement of synaptic connections

correlated electrical activity had to occur with in a narrow time window [379].

5.3.4. Mammals

Several lines of evidence exist that chickens and mammals obey similar mechanisms of retinotectal development. The recombinant human Slit-2 results in collapse of chicken retinal growth cones [248]. Slit-2 is also expressed in the eye, in the optic stalk, and in the ventral diencephalon in the mouse and uses the putative receptors robo1 and robo2 which are expressed in the RGC-layer. Another example of molecules found in many species are ephrins [16,25]. There is no doubt that conserved, species-independent molecules will be further discovered to finally unravel the exact cascades of axonal pathfinding which operate in a similar way perhaps in all vertebrates. Disease-associated genes of the Pax-family cause a number of eye abnormalities in humans like the aniridia-syndrome or microphthalmia [200].

The most successful methodological approach in contemporary developmental biology employs tools of molecular biology to identify new genes. Perhaps the most wide-spread strategy to demonstrate functional effects of developmental genes consists in the production of transgenic animals that lack or overexpress the genes in question. This has to be followed up by careful analyses of the developmental defects. The overwhelming number of these studies is conducted with mice. While the present review is testimony for the chick visual system being still a successful model in the investigation of axonal pathfinding and connectivity, the invention of the 'knockout-mouse' has provided a technical advantage for the mouse as a model organism. Consequently a growing number of experiments on visual system development is conducted in mammals [34,104].

5.3.5. Other birds

In the area of avian electrophysiological research pigeons rather than chickens have been traditionally the species of choice. The representation of the pigeon retina on the tectum was studied by Hamdi and Whitteridge [147] and is identical with the representation in the chicken [216]. Being an altricial bird species pigeons differ from chickens in the time-course of retinotectal development, and in the fact that several characteristic features of visual connectivity develop in the post-hatching period [164,165]. Such features are the maturation of optic terminal profiles [1], the retraction of ipsilateral fibres and morphological-functional maturation [8]. Due to the regional specialisation of the retina, similar regional differences are inferred to the pigeon optic tract, chiasm and retinoreceptive layers of the tectum [79]. One marked difference from the chicken is the pigeon-specific change of visual acuity [155] and of electroretinographic performance with age [265].

Since it became possible to successfully transplant tissues between quail and chick embryos and to distinguish cells from the two species, a considerable amount of attention has been paid to the quail visual system that shows similar phenotypic development with the chick. However, development is faster with an embryonic incubation of 16–17 days instead of 20–21 days in the chick. The quail was mainly used to perform transplantations of retinal or tectal primordia and create chimeric animals [2,3]. The basic mechanisms of axonal guidance in the quail were studied by Halfter and Deiss [135,136] and Halfter and Schurer [137]. According to the work of Senut and Alvarado-Mallart [2,3], there is no difference in the intrinsic organisation between the chick and quail retinotectal pathways. With cellular markers that are available to identify the quail cells from those in the chick primordial grafting between the two species further helped to define the molecular boundaries between the mesencephalon and the hindbrain region [153,154].

6. Conclusion

The endeavour to understand the mechanisms of retinal axonal guidance over long distances and local navigation within a target area requires a synthesis of many methodological approaches. This has been accomplished in the retinotectal projection of the chick. The approaches include classical embryology, in vitro models, neuroanatomical tools, molecular, probes and invasive methods ranging from tissue transplantation to gene targeting with viral vectors.

As a conclusion, we briefly summarise the present knowledge about this system (Fig. 9).

(1) The positional determination of retinal axes occurs at E2 (HH-11 and before) in the early stages of eye formation. At this time axial polarity is established by asymmetrically distributed transcription factors like CBF-1, CBF-2, engrailed, Tbx-5 and Pax-2, which imprint neuronal stem cells in the retina with an permanent mark of positional identity. Although the entire cascade of molecular interactions is not understood, the specification of cellular identity within the retina (and tectum) is a fundamental and instructive mechanism, some key players of which have been identified. CBF-1 and CBF-2 determine the pattern of axonal marking along the naso-temporal axis. BMP-4, Tbx-5, Vax and possibly retinoic acid seem to convey positional identity along the dorso-ventral direction. Patterning of the complementary tectal axial polarity is controlled by the transcription factors Otx-2, Gbx-2, Wnt-1 and the *engrailed* proteins. The downstream rostro-caudal asymmetry of ephrins and Eph receptors within the tectum is triggered by the rostro-caudal gradient of *engrailed*.

(2) Guidance of axons over long-distance, that is from the molecularly specified position of outgrowth through the intraretinal pathway, the optic stalk (nerve), the chiasm and the optic tract obeys different molecular rules. Within the

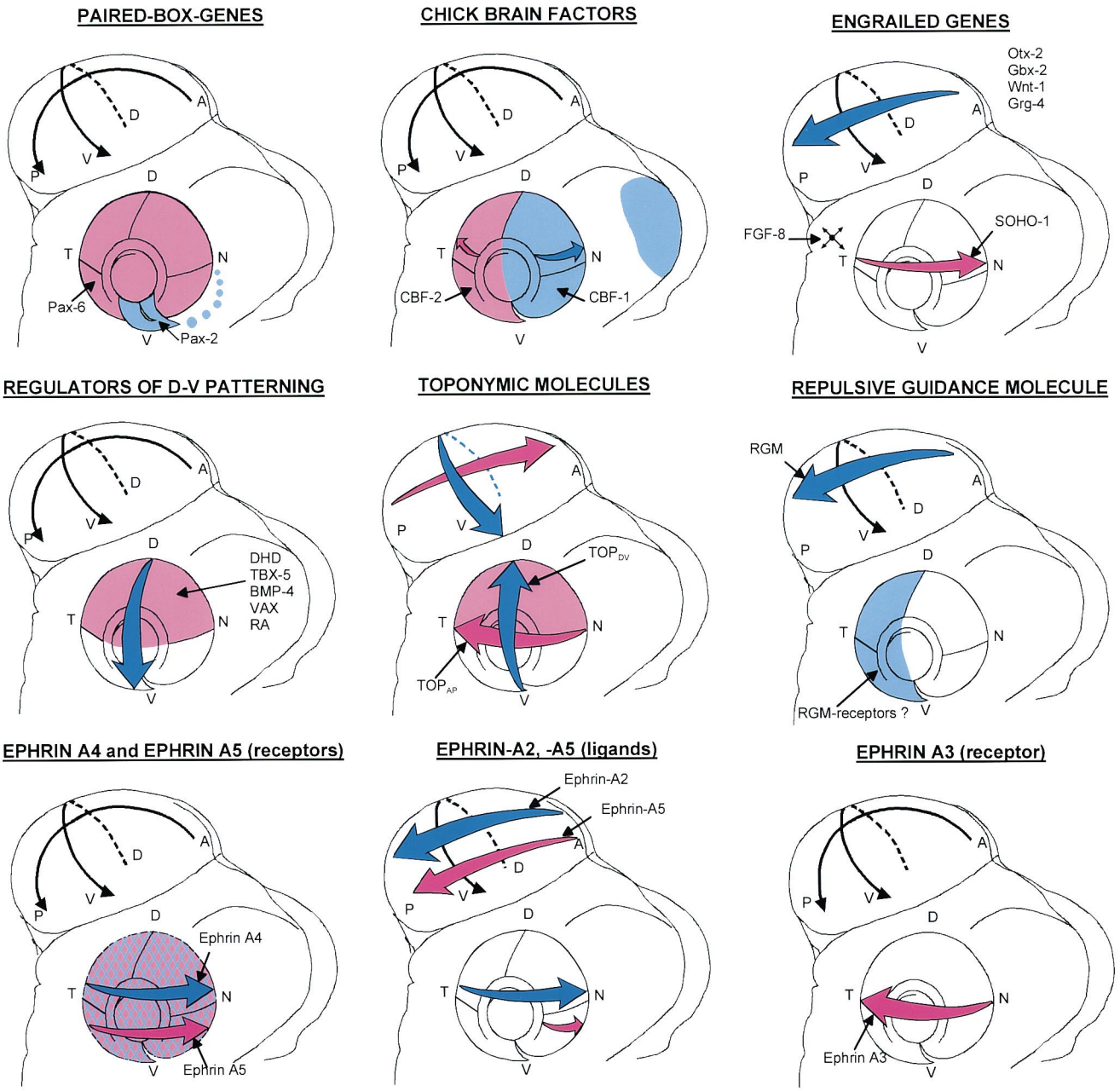

Fig. 9. Schematics summarizing the distribution of different genes, transcription factors and surface molecules proposed to be involved in the formation of the retinotectal topography. Whereas Pax-2 and Pax-6 are involved in eye development, the transcription factors CBF-1 and CBF-2 are determined by the naso-temporal specificity. At the same time tectal specification is controlled by several genes. Emerging ligand–receptor interactions are drawn for the ephrins. Increased thickness of the arrows indicates increasing gradients, whereas different colours are used to outline co-expressions and boundaries both in the eye and the tectum. Details on each molecule are described in the text.

retina, polarisation of the differentiating RGC manifests itself morphologically by the formation of a vitreal end-foot. This is a cellular protrusion which remains in contact with laminin-containing basal membrane where it develops into an axonal growth cone. The molecular outfit of this growth cone implies the essential spatial positional information (e.g. ephrin and Eph receptors, CBF-controlled downstream molecules, RPTPs like CRYPα, RA-receptors), and the equipment of growth associated receptors (integrins, cell adhesion molecules, neurotrophin receptors and GAPs). This molecular panoply enables the growth cone to interact with environmental guidance cues from the environment. Within the retina it traverses towards the optic fissure and exits into the optic stalk.

(3) Since the environment changes qualitatively and quantitatively within the optic stalk (nerve), the molecular set of positional identity may remain, but the growth-associated set of molecules may be changed or redistri-

buted to meet the environmental demands. There are no other retinal cells, different glial endfeet, different arrangements of axons and so on. The cataclysmic death of primitive glial cells in the supraoptic area is perhaps associated with the directional growth through the chiasm midline, and the changes of growth direction immediately after crossing to the contralateral side (optic tract). Although not studied in detail, the midline that forms the presumptive chiasm may be as complex as the CNS-midline that is decorated by a set of molecules involved in growth cone guidance. The multilayered chiasm, which consists of more than 30 tiers of alternating origin between the two retinas, suggests the presence of specific mechanisms for preserving positional identity. Immediately after crossing, the bundle of axons becomes flattened to form the optic tract.

(4) Target-dependent guidance of axons is assumed to become relevant within the diencephalon (optic tract) where the di-mesencephalic boundary is controlled by *engrailed*. This protein, whose decreasing caudal-rostral gradient is triggered by FGF-8, causes the asymmetric expression of ephrins and Eph receptors. It is therefore plausible that retinal growth cones, whose membranes display ephrins and Eph receptors, can read the gradients of molecules in the environment. The high-nasal-to-low-temporal gradient of Eph-A ligands determines the growth cone sensitivity to tectal receptors in a diametrically opposed gradient. Misexpression of EphA-2 in the retina leads to topographical errors along the rostro-caudal axis.

(5) The intratectal guidance of axons seems to be conveyed by graded molecules including transcription factors, secreted and, mostly, membrane-bound receptors and ligands (Fig. 9). However, we understand little of the entire system of these molecules and their hierarchical interplay. Also the steepness of the gradients is unclear and may change over development. From E7 to E12–13 axons advance over the tectal surface, then dive into the SGFS and connect with corresponding tectal neurons. The area of innervation covered by terminals from a local point in the retina condenses during a period of refinement that is due to retraction of inappropriate terminals and/or cell death. Neuronal activity provides mechanisms for this refinement at later embryonic stages, which have not been dealt with in this review. At the day of hatching the chicken visual system is considered mature.

(6) Out of the models that have been developed to predict and explain the experimental data, the chemoaffinity hypothesis, based on gradients of cell surface molecules has gained the most attraction and has been corroborated by the most empirical data. It remains, however, an ongoing discussion whether more repulsion and less chemoattraction or vice versa is causally involved in the complex network of map formation.

In this review, we have drawn together data from many in vitro studies and a wide variety of in vivo experiments and observations, all done in the chick embryo, to gain an understanding of the mechanisms that create a topographic retinotectal connection. After more than 100 years of experimental research on development of the vertebrate visual system the following questions stand out:

To what degree do the several guidance mechanisms discovered in vitro become relevant in retinotectal pathfinding? How do they interact with each other?

What is the precise translation from external guidance cues to the directed growth movements of axonal growth cones? What is the hierarchy of genes and molecules involved and what are the signal transduction mechanisms?

How does the long-distance control of ordered growth differ from short-range intratectal refinement of growth? Finally, are the guidance molecules repellent or attractive to axonal growth cones?

7. Uncited references

[36], [41A], [62], [116], [176], [182], [184], [189], [201], [205], [209], [233], [234], [297], [298], [302], [305], [311]

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